Response to Reviewer #2:

Comment 1:

The review should be re-structured to improve readability. It might be beneficial to incorporate more subheadings or to re-structure according to HNCC subsides.

Authors' reply:

Thank you for the suggestions. We re-arrange the paragraph of 4.1. miRNAs as tumor suppressors to improve the readability. Since the 4.1 section contains massive information, we reorganized the manuscript and added the subheadings as the reviewer's suggestion based on the HNSCC subtypes, as 4.1.1 miRNAs in HNSCC; 4.1.2 miRNAs in OSCC; 4.1.3 miRNAs in LSCC; 4.1.4 miRNAs in NPC.

Comment 2:

From a clinical perspective: The subdivision into "tongue SCC" doesn't make sense at all. The clinical and pathological TNM classification distinguishes SCCs from tongues into either oropharyngeal SCCs (base of tongue) or oral SCCs (rest of tongue).

Authors' reply:

We appreciate your suggestion and revised our manuscript more precisely as below.

In line 54-58 of the revised manuscript without track changes (line 75-79 with track changes):

HNSCCs subtypes include oral SCCs (OSCCs), larynx SCCs (LSCCs), nasopharyngeal carcinomas (NPCs), and oropharynx SCCs (OPSCCs) [5,6]. It should be noted that with tongue squamous cell carcinomas, an OSCC includes the anterior two-thirds of the tongue (anterior to the circumvallate papillae) and an OPSCC consists of the base (or posterior one-third) of the tongue [7].

In line 253-255 of the revised manuscript without track changes (line 1026-1028with track changes):

EZH2 was shown to promote cell migration, invasion, and metastasis, and the EMT, thereby enhancing cellular plasticity for oral tongue squamous cell carcinomas [133,134].

In line 306-307 of the revised manuscript without track changes (line 1158-1160 with track changes):

In OSCCs and OPSCCs, *miR-34a* is described as a regulator of SCs [159].

In line 327-330 of the revised manuscript without track changes (line 1587-1590 with track changes):

miR-22 inhibits phosphatidylinositol 3-kinase (PI3K)/Akt/NF-κB signaling via downregulating activators such as *S100A8*, platelet-derived growth factor (*PDGF*), and vascular endothelial growth factor (*VEGF*), which implies a tumor suppressor role of

miR-22 in tongue squamous cell carcinoma [168].
In line 344-345 of the revised manuscript without track changes (line 1604-1605 with track changes):
Qiu et al. indicated that the downregulation of *miR-22* would result in the upregulation of CD147 in tongue squamous cell carcinomas [190].
In line 374-375 of the revised manuscript without track changes (line 1730-1731 with track changes):
By downregulating *ATF3* expression, *miRNA-488* suppresses cell invasion and the EMT in tongue squamous cell carcinoma cells [206].
In line 424-426 of the revised manuscript without track changes (line 1780-1896 with track changes):
miR-19a and *miR-424* inhibit the TGF-β type III receptor (*TGFBR3*), also known as β-glycan, which results in promoting the migration and EMT of tongue squamous carcinoma cells [98].

Comment 3:
Line 60/61: This sentence is not correct as it is: Radiotherapy and chemotherapy alone are no therapeutic options for HNSCCs except for palliative treatment. Chemotherapy is usually combined with radiotherapy in an adjuvant setting.
Authors' reply:
Thank you for the comment. There is a different treatment choice variation based on the region of HNSCC and the clinical staging. It can be surgery combined with radiotherapy, surgery combined with chemoradiotherapy, radiotherapy alone (Machiels et al., 2020, ref 11 in the revised manuscript), and chemoradiotherapy. We modified the sentence about those treatment options as below.
In line 66-68 of the revised manuscript without track changes (line 87-89 with track changes):
Therapeutic approaches include dissection, radiotherapy, chemoradiotherapy, and a combination of dissection with radiotherapy or chemotherapy. Chemoradiotherapy may be taken as adjuvant therapy in advanced stages [11].

Comment 4:
Table 1: miR-205-5p and miR-1972 are missing in the list and text. Lately, there has been evidence that these two could be exosomal markers for HPV+/- HNSCCs: miR-205-5p in HPV(+) and miR-1972 in HPV(-) exosomes (PMID: 33202950)
Authors' reply:
Thank you for the suggestions. In the manuscript Ref [162], Gregory et al. indicated that miR-205 and the miR-200 family regulate EMT by targeting ZEB1 and SIP1 in

breast cancer. Consistently, other papers also supported the role of the miR-200 family in regulating EMT and stemness in HNSCC. miR-205 regulates the cancer stemness has been shown in breast cancer **(Plantamura et al., 2020; Xiao et al., 2019)**, prostate cancer **(Ferrari and Gandellini, 2020; Gandellini et al., 2009; Tucci et al., 2012)**, colorectal cancer **(Gulei et al., 2018)**, and pancreatic cancer **(Chaudhary et al., 2017)**, instead of HNSCC. Moreover, the evidence of miR-1972 in regulating cancer stemness has not been addressed. Therefore, in the previous version, we did not include the information about these two miRNAs since our goal is to summarize the stemness regulating miRNAs in HNSCC.

In this revised version, we edit the miR-205 and miR-200 family descriptions in regulating EMT as below. Moreover, we think this paper **(PMID: 33202950)** is fascinating. We incorporated it into the conclusion section to discuss the potential of HPV and exosomal miRNAs in regulating HNSCC stemness as below.

In line 313-318 of the revised manuscript without track changes (line 1166-1171 with track changes):

Gregory et al. first indicated that miR-205 and the *miRNA-200* family (*miR-200a*, *miR-200b*, *miR-200c*, *miR-141*, and *miR-429*) suppressed the EMT by targeting zinc finger E-box binding homeobox 1 (*ZEB1*) and Smad-interacting protein 1 (*SIP1*, also known as *ZEB2*) in breast cancer [162]. Similarly, the miRNA-200 family was indicated to enhance EMT through a reciprocal feedback loop between the miR-200 family and ZEB1 in HNSCCs [163,164].

In line 455-482 of the revised manuscript without track changes (line 1925-1977 with track changes):

Moreover, with an understanding of miRNAs during tumorigenesis, we can take advantage of miRNA stability and use it as a diagnostic marker for primary diagnoses and patient follow-ups. We can also monitor miRNA changes to predict therapeutic responses as a non-invasive detection method. Recent studies have indicated that exosomal miRNAs can be better sources of biomarkers due to their advantages in terms of their quantity, quality, and stability [239]. Ludwig et al. indicated that miR-205-5p was exclusively expressed in HPV(+) exosomes, whereas miR-1972 was only detected in HPV(−) exosomes. These miRNAs emerge as potential discriminating HPV-associated biomarkers [240]. Intriguingly, human papillomavirus 16 (HPV16) infection has been indicated to enhance CSC properties, including ALDH1 activity, migration/invasion, and CSC-related factor expression, and enhances tumor growth OSCC cells [241]. Whether tumor-derived exosomes (TEX)-miRNAs are also involved in regulating the recipient cell stemness is unclear. In contrast to the extensive studies

for cellular miRNAs in regulating cancer stemness, TEX-miRNA knowledge is relatively limited.

Moreover, Huang et al. indicated that only 5.63% of miRNAs were detected in both cells and TEX, which implies that cells can selectively pack certain miRNAs into exosomes in OSCC cells [242]. Meanwhile, exosomes can be released by various cell types, such as cancer-associated fibroblasts (CAFs) [243], dendritic cells [244], B cells [245], T cells [246], and tumor cells [247]. For example, Li et al. indicated that miR-34a-5p was significantly reduced in CAF-derived exosomes in OSCC patients. CAF transfers miR-34a-5p-devoid exosomes to OSCC cells and results in promoting the proliferation and motility of OSCC cells by upregulating the downstream target AXL (encoding AXL receptor tyrosine kinase). Therefore, the miR-34a-5p/AXL axis promotes the proliferation, metastasis, and EMT of oral cancer cells through the AKT/glycogen synthase kinase (GSK)-3β (GSK-3β)/β-catenin/Snail signaling cascade [248]. Consistently, the cellular miR-34a significantly inhibited EMT formation of the CSC-phenotype in HNSCC cell lines [161]. Hence, the sources and biological functions of exosomal miRNAs warrant further research before using them for screening and surveillance.

Comment 5:

Formal issue: Genes should be italicized.

Authors' reply:

Thank you for the comment. We had revised the manuscript.

Comment 6:

The authors should consider English editing services: There are some spelling issues (i.e. Line 1: 1 is missing, Line 83: survive instead of survival, Line 95: the word marker is doubled, Line 197(199): miRNAs that are involved.../involved (no capital letter), Line 219/20(323): hyphen is missing in miR-34a, Line 360(463): metastatic instead of metastasis).

Authors' reply:

We have revised the sentence according to the suggestion. We also send the manuscript for English editing. The certificate of English editing is attached at the bottom of the letter.

Reference:

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We certify that the following article

Roles of microRNAs in Regulating Cancer Stemness in Head and Neck Cancers

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has undergone English language editing by MDPI. The text has been checked for correct use of grammar and common technical terms, and edited to a level suitable for reporting research in a scholarly journal.
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