Plasma Extracellular Vesicles-Derived miR-99a-5p: A Potential Biomarker to Predict Early Head and Neck Squamous Cell Carcinoma

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Purpose: This study aimed to investigate the applicability of plasma extracellular vesicles (EVs) miR-99a-5p as a potential head and neck squamous cell carcinoma (HNSCC) diagnostic biomarker.

Methods: The miRNA expression of HNSCC tissue and plasma EVs were profiled by small RNA sequencing. qRT-PCR was performed to detect miR-99a-5p expression in HNSCC (n = 93) and benign disease (n = 39) plasma EVs and formalin-fixed and paraffin-embedded (FFPE) tissue (n = 110). We constructed receiver-operating characteristic curves to investigate the diagnostic efficiency of plasma EVs miR-99a-5p.

Results: Tumor tissue exhibited lower miR-99a-5p than para-tumor tissue. Patients with high miR-99a-5p expression exhibited significantly more p16 positive status. In contrast, HNSCC plasma EVs harbored more miR-99a-5p than the benign disease group. Plasma EVs miR-99a-5p distinguished HNSCC with area under the curve (AUC) of 0.7494 (95% CI: 0.6692–0.8296; p < 0.0001), with 61.54% sensitivity and 75.27% specificity, respectively. Furthermore, plasma EVs miR-99a-5p also distinguished early HNSCC with AUC of 0.7394 (95% CI: 0.6284–0.8504; p = 0.0002), with 79.07% sensitivity and 61.54% specificity, respectively.

Conclusion: Plasma EVs miR-99a-5p is a potential biomarker for predicting early HNSCC.

Keywords: biomarker, HNSCC, HPV, plasma extracellular vesicles, miR-99a-5p

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC), a heterogeneous collection of malignancies, accounts for approximately 90% of all head and neck cancers and is closely associated with human papilloma virus (HPV) infection [1]. The epidemiology, pathophysiology, and response to treatment of HPV+ HNSCC differ sharply from that of HPV− disease. Overall, HPV positive is associated with more favorable clinical outcomes [2]. Notably, HNSCC patients are at a high risk of cervical lymph node metastases. Cervical lymph node involvement is a well-known prognostic marker for HNSCC [3], and the presence of positive lymph nodes is thought to be a
Plasma samples were obtained from HNSCC patients ([formalin-fixed and paraffin-embedded (FFPE) HNSCC tissue (n = 110) were obtained from patients diagnosed with HNSCC (mainly hypopharyngeal and laryngeal squamous cell carcinoma) pathologically after surgery from October 2019 to October 2021. Plasma samples were obtained from HNSCC patients (n = 93) and benign disease patients (n = 39) from October 2019 to October 2021. All patient samples were obtained from the Department of Otorhinolaryngology, Eye & ENT Hospital of Fudan University. All participants provided written informed consent forms. This study was approved by the Ethics Committee of the Eye & ENT Hospital of Fudan University (NO. 2018036).

Methods and Materials

Patient Tissue and Ethics Approval
HNSCC tumor and para-tumor fresh tissue (n = 3 pairs) and formalin-fixed and paraffin-embedded (FFPE) HNSCC tissue (n = 110) were obtained from patients diagnosed with HNSCC (mainly hypopharyngeal and laryngeal squamous cell carcinoma) pathologically after surgery from October 2019 to October 2021. Plasma samples were obtained from HNSCC patients (n = 93) and benign disease patients (n = 39) from October 2019 to October 2021. All patient samples were obtained from the Department of Otorhinolaryngology, Eye & ENT Hospital of Fudan University. All participants provided written informed consent forms. This study was approved by the Ethics Committee of the Eye & ENT Hospital of Fudan University (NO. 2018036).

RNA Isolation and qRT-PCR
Total RNA was isolated from FFPE tissue with an RNeasy FFPE Kit (QIAGEN, Germany) and from fresh tumor tissue and plasma EVs with TRIzol reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer’s instructions, measured by a microspectrophotometer Nanodrop 2000 (Thermo Fisher Scientific), with the ratio of OD260/OD280 > 1.8, and then reversed-transcribed using an Evo M-MLV Mix Kit with gDNA Clean for qPCR (AG11728, Accurate Biology, Hunan, China). The housekeeping genes U6 and RNU6 were used as internal references to normalize gene expression for miRNA. The primers were designed and synthesized by Sangon Biotech (Shanghai). The sequences of primers used are as follows: miR-99a-5p: 5′-AACCCGTAGATCCGATCTTGTG3′; U6: 5′-GTTCTCGCTTGCGACGACAT3′. qRT-PCR was performed using SYBR® Green Premix Pro Taq HS qPCR Kit (AG11718, Accurate Biology, Hunan, China) using the 2−ΔΔCt method.

Isolation and Purification of EVs
Peripheral blood samples were obtained in an ethylene diamine tetraacetic acid (EDTA)-coated tube (BD Phamriningen, New Jersey, USA) before surgery. Hemocytes were separated by centrifugation at 2,000 g for 15 min at 4°C. The clear top layer was obtained by another centrifugation at 10,000 g for 30 min at 4°C. The EVs derived from the plasma samples were isolated with Total Exosome Precipitation Reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer’s instructions.

Confirmation and Characterization of Plasma EVs
The morphological characteristics, size distributions, and marker detection of EVs pellets were examined by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and immunoblotting analysis, according to the methods described in our previous studies [16, 17].

Small RNA Sequencing and Data Analysis
Small RNA library preparation and sample sequencing were performed with the assistance of Beijing Novogene Co., Ltd., using an Illumina HiSeq™ 2500 device. Total RNA from fresh HNSCC and para-tumor tissue (n = 3 pairs) and plasma EVs (n = 6 for HNSCC patients, n = 3 for benign disease patients) were concatenated with 5′ and 3′ adaptors. The quantity and integrity of RNA yield was assessed by using the Qubit®2.0 (Invitrogen, USA) and Agilent 2200 TapeStation (Agilent Technologies, USA) separately. After cDNA synthesis and PCR amplification, the cDNA library (18–40 nt) was obtained using an acrylamide gel purification method, and single-end sequencing was then performed. The raw data (Raw reads) obtained by sequencing were filtered first: the joints at both ends of the reads were removed, and the reads with fragment length <17 nt and low-quality reads were removed to complete the preliminary filtering of data and obtain high-quality data (Clean reads). The distribution map of genome-wide reads was obtained by comparing Clean reads with the reference genome, and the Clean reads were annotated by ncRNA classification. The miRNA expression was calculated by RPM (Reads Per Million) values (RPM=(number of reads mapping to miRNA/number of reads in Clean data)×10⁶). Differential expression between two sets of samples was calculated by edgeR algorithm according to the criteria of |log2(Fold Change)|≥1 and p-value < 0.05.
Bioinformatics Analysis
The expression of miR-99a-5p was downloaded from the latest TCGA project (Data Release 32.0) via the Genomic Data Commons Data Portal [18] (https://portal.gdc.cancer.gov/). The expression values of genes from miRNA-seq data were scaled with log2(RPM +0.01). A Kaplan–Meier plot was used to assess the correlation between the expression of miR-99a-5p in HNSCC.

Statistical Analysis
Statistical analysis was performed using the Mann–Whitney test to evaluate the differences between the two groups. The Chi-square testing was used for the statistical calculation of categorical data. Kaplan-Meier method was used to calculate the survival rate, and Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test were used to test the difference in survival rate. GraphPad Prism 8.0 was used for statistical analyses and scientific graphing. The results are expressed as mean ± standard deviation (SD). Receiver-operating characteristic (ROC) curves were performed by plotting the true positive (sensitivity) against the false-positive (1-specificity) rate, and the area under the curve (AUC) was calculated. Optimal cut-off values were established based on the highest value obtained in the ROC curve analysis according to the likelihood ratio. Differences were considered significant if the p value was <0.05 (*), <0.01(**), <0.001(***), or <0.0001 (***) as indicated in each figure legend.
RESULTS

miR-99a-5p Is Downregulated in HNSCC Tissue and Is Associated With HPV Infection Status

To depict the comprehensive miRNA profile with abnormal expression, miRNA sequencing was performed in three paired HNSCC tumors and para-tumor tissue. Differentially expressed miRNAs are listed in Figure 1A. miRNA-99a-5p was consequently selected for further study due to its high expression in para-tumors but low expression in HNSCC tumors (Figure 1B). Moreover, we verified the expression of miR-99a-5p in the Cancer Genome Atlas (TCGA) cohort. Data from 44 normal tissues and 497 HNSCC tumors confirmed that miR-99a-5p expression was higher in normal tissues than in tumor tissues, which was consistent with the sequencing results (Figure 1C). Furthermore, the Kaplan–Meier plot showed that low expression of miR-99a-5p in HNSCC tissues negatively affected the prognosis of HNSCC patients (p < 0.05) (Figure 1D).

We further determined miR-99a-5p expression in 110 HNSCC FFPE tissue using qRT-PCR. Detailed clinical and pathological data are depicted in Table 1. Overall, patients with high miR-99a-5p expression exhibited significantly more p16 positive status in pathological samples than did patients with low miR-99a-5p expression (p < 0.0001). No further significant associations were found.

### Table 1: Correlation between miR-99a-5p and clinicopathologic characteristics in 110 HNSCC FFPE tissue.

| Parameter                          | All patients | High expression | Low expression | p value |
|------------------------------------|--------------|-----------------|----------------|---------|
|                                    | n = 110      | n = 22          | n = 88         |         |
| Age                                |              |                 |                |         |
| ≤60                                | 60           | 15              | 45             | 0.1510  |
| >60                                | 50           | 7               | 43             | >0.9999 |
| Gender                             |              |                 |                |         |
| Female                             | 1            | 0               | 1              | >0.9999 |
| Male                               | 109          | 22              | 87             | 0.3940  |
| Smoking history                    |              |                 |                |         |
| No                                 | 25           | 3               | 22             | 0.1247  |
| Yes                                | 85           | 19              | 66             |         |
| Drinking history                   |              |                 |                |         |
| No                                 | 35           | 10              | 25             | 0.6971  |
| Yes                                | 75           | 12              | 63             |         |
| Hypertension                       |              |                 |                |         |
| No                                 | 66           | 14              | 52             | 0.2253  |
| Yes                                | 44           | 8               | 36             |         |
| Diabetes                           |              |                 |                |         |
| No                                 | 99           | 18              | 81             | 0.0001* |
| Yes                                | 11           | 4               | 7              |         |
| p16                                |              |                 |                |         |
| Negative                           | 92           | 7               | 85             | 0.8453  |
| Positive                           | 18           | 15              | 3              |         |
| T stage                            |              |                 |                |         |
| T1+T2                              | 52           | 10              | 42             | 0.9999  |
| T3+T4                              | 58           | 12              | 46             |         |
| Lymph node metastasis              |              |                 |                |         |
| Negative                           | 20           | 4               | 16             | >0.9999 |
| Positive                           | 90           | 18              | 72             |         |
| Clinical stage                     |              |                 |                |         |
| I + II                             | 7            | 1               | 6              | 0.5010  |
| III + IV                           | 103          | 21              | 82             |         |
| Maximum tumor diameter (MTD)       |              |                 |                |         |
| ≤3 cm                              | 62           | 11              | 51             | 0.3670  |
| >3 cm                              | 48           | 11              | 37             |         |
| Differentiation                    |              |                 |                |         |
| High+ High-moderate                | 92           | 17              | 75             | 0.3880  |
| Moderate+ moderate-low             | 18           | 5               | 13             |         |
| Recurrence                         |              |                 |                |         |
| No                                 | 61           | 14              | 47             | 0.3811  |
| Yes                                | 49           | 8               | 41             |         |
| Death                              |              |                 |                |         |
| No                                 | 66           | 15              | 51             |         |
| Yes                                | 44           | 7               | 37             |         |

*p value was tested from Fisher’s exact test.

From 44 normal tissues and 497 HNSCC tumors confirmed that miR-99a-5p expression was higher in normal tissues than in tumor tissues, which was consistent with the sequencing results (Figure 1C). Furthermore, the Kaplan–Meier plot showed that low expression of miR-99a-5p in HNSCC tissues negatively affected the prognosis of HNSCC patients (p < 0.05) (Figure 1D). We further determined miR-99a-5p expression in 110 HNSCC FFPE tissue using qRT-PCR. Detailed clinical and pathological data are depicted in Table 1. Overall, patients with high miR-99a-5p expression exhibited significantly more p16 positive status in pathological samples than did patients with low miR-99a-5p expression (p < 0.0001). No further significant associations were found.
between miR-99a-5p levels in HNSCC tissue and clinicopathological features (T stage, lymph node metastasis, clinical stage, tumor diameter, or pathological differentiation). We also found no difference in tissue miR-99a-5p levels in terms of prognosis (p = 0.3880) or recurrence (p = 0.3881) in HNSCC patients.

Collectively, these findings suggest that miR-99a-5p may play an important role in inhibiting the progression of HNSCC and is associated with HPV infection status.

miR-99a-5p is Upregulated in HNSCC Plasma EVs

Considering the promising results obtained in tissue samples and the significant anti-tumor properties of miR-99a-5p previously described in HNSCC [19], we hypothesized that the tumor excreted cancer-suppressing miR-99a-5p into the peripheral circulation in the form of EVs to maintain survival [20]. To confirm this hypothesis and further describe the miRNA expression profile in plasma EVs of HNSCC patients, we isolated EVs from the plasma of six HNSCC patients and three benign disease patients, and then performed miRNA sequencing.

We found that the EVs we isolated had a representative saucer-shaped vesicle structure with a double-layer membrane, according to the TEM analysis (Figure 2A). We further verified the expression of EVs surface markers CD63 and CD9, together with the negative marker Calnexin. Furthermore, plasma EVs did not express platelet and immune cell marker genes CD41 and CD11b (Figure 2B). The NTA results showed that the average size of the EVs was 157.6 ± 68.7 nm for EVs derived from patients with benign disease and 161.6 ± 58.6 nm for HNSCC patients-derived EVs (Figure 2C). These results confirmed that the EVs we isolated had representative morphology, appropriate size distribution, and precise surface markers typical of EVs.

Further, we identified several miRNAs, including miR-1228-5p and miR-4466, that were highly expressed in the plasma EVs of HNSCC patients through miRNA sequencing. Clinicopathological features of HNSCC patients undergoing plasma EVs miRNA sequencing are summarized in Table 2. Surprisingly, we found that, contrary to the miR-99a-5p level in
tissue, miR-99a-5p expression levels in circulating plasma EVs were significantly higher in HNSCC patients than in benign disease controls (Figure 2D).

The unexpected opposite trend in tissue and plasma EVs suggests that circulating plasma EVs miR-99a-5p may be a potential biomarker for the detection of HNSCC.

TABLE 2 | Clinicopathological features of HNSCC patients undergoing plasma EVs miRNA sequencing.

| No. | Gender | Age | Tumor type | T stage | N stage | Clinical stage | Pathological differentiation | p16 status |
|-----|--------|-----|------------|---------|---------|---------------|-----------------------------|------------|
| 1   | Male   | 73  | LSCC       | 3       | 1       | III           | Well-moderately             | Positive   |
| 2   | Male   | 74  | LSCC       | 3       | 1       | III           | Well-moderately             | Positive   |
| 3   | Male   | 64  | HPSCC      | 4       | 2       | IV            | Poor                        | Positive   |
| 4   | Male   | 68  | HPSCC      | 4       | 1       | IV            | Well-moderately             | Negative   |
| 5   | Male   | 65  | HPSCC      | 3       | 1       | III           | Poor                        | Negative   |
| 6   | Male   | 69  | HPSCC      | 3       | 2       | IV            | Poor                        | Positive   |

LSCC, laryngeal squamous cell carcinoma; HPSCC, hypopharyngeal squamous cell carcinoma.

FIGURE 3 | miR-99a-5p as a biomarker for early HNSCC detection. (A) miR-99a-5p expression in HNSCC patients (n = 93) compared with that in benign disease patients (n = 39) detected by qRT-PCR. U6 serves as an internal control. Mann–Whitney test, ****p < 0.0001. (B) Diagnostic potential of plasma EVs miR-99a-5p in HNSCC patients. The area under the ROC curve was 0.7494 (95% CI: 0.6692 to 0.8296; p < 0.0001). (C) miR-99a-5p expression in early HNSCC patients (n = 43) compared with that in benign disease patients (n = 39) detected by qRT-PCR. U6 serves as an internal control. Mann–Whitney test, ***p = 0.0001. (D) Diagnostic potential of plasma EVs miR-99a-5p in early HNSCC patients. The area under the ROC curve was 0.7394 (95% CI: 0.6284 to 0.8504; p = 0.0002). The results are expressed as mean ± SD.
miR-99a-5p as a Biomarker for Early HNSCC Detection

To further confirm the hypothesis previously proposed, we explored the diagnostic value of plasma EVs miR-99a-5p as a minimally invasive HNSCC diagnostic biomarker. We isolated plasma EVs from HNSCC patients (n = 93) and benign disease patients (n = 39) and assessed the miR-99a-5p expression level by qRT-PCR. We found that miR-99a-5p expression levels were significantly higher in plasma EVs from HNSCC patients (median: 1.445) than in those from benign disease patients (median: 0.7687) (p < 0.0001) (Figure 3A). Detailed clinical and pathological data on 93 HNSCC patients are presented in Table 3. Overall, patients with high plasma EVs miR-99a-5p expression were significantly younger (p = 0.0129) and had a lower p16 positive status in pathological samples (p = 0.0134) than those with low expression. No further significant associations were found between miR-99a-5p levels in HNSCC plasma EVs and clinicopathological features (T stage, lymph node metastasis, clinical stage, tumor diameter, or pathological differentiation).

To assess the potential value of plasma EVs miR-99a-5p for the diagnosis of HNSCC, ROC curves were constructed to distinguish HNSCC patients from benign disease patients (Figure 3B). The AUC was 0.7494 (95% CI: 0.6692–0.8296; p < 0.0001). When the optimal cut-off value was 0.8537, the sensitivity and specificity were 61.54% (0.4590–0.7511) and 75.27% (0.6562–0.8292), respectively.

Given the current lack of effective biomarkers for early HNSCC, we further explored whether plasma EVs miR-99a-5p could be a biomarker for early HNSCC detection. We found that early HNSCC patients (n = 43) had higher expression levels of miR-99a-5p in plasma EVs than benign disease patients (p = 0.0001) (Figure 3C). Plasma EVs miR-99a-5p expression level was able to discriminate early HNSCC from benign disease patients with an AUC of 0.7394 (95% CI: 0.6284–0.8504; p = 0.0002). Furthermore, using the cut-off value mentioned above,

### Table 3: Correlation between plasma EVs miR-99a-5p and clinicopathologic characteristics in 93 HNSCC patients.

| Parameter                  | All patients | High expression | Low expression | p value |
|----------------------------|--------------|-----------------|----------------|---------|
|                            | n = 93 | %              | n = 38 | %         | n = 55 | %         |
| Age                        |              |                 |              |          |          |          |
| ≤60                        | 22         | 23.7            | 14         | 36.8     | 8       | 14.5     |
| >60                        | 71         | 76.3            | 24         | 63.2     | 47      | 85.5     |
| Gender                     |              |                 |              |          |          |          |
| Female                     | 3           | 3.2             | 3           | 7.9      | 0       | 0        |
| Male                       | 90          | 96.8            | 35          | 92.1     | 55      | 100      |
| Smoking history            |              |                 |              |          |          |          |
| No                         | 25          | 26.9            | 10          | 26.3     | 15      | 27.3     |
| Yes                        | 68          | 73.1            | 28          | 73.7     | 40      | 72.7     |
| Drinking history           |              |                 |              |          |          |          |
| No                         | 34          | 36.6            | 12          | 31.6     | 22      | 40       |
| Yes                        | 59          | 63.4            | 26          | 68.4     | 33      | 60       |
| Hypertension               |              |                 |              |          |          |          |
| No                         | 50          | 53.8            | 19          | 50       | 31      | 56.4     |
| Yes                        | 43          | 46.2            | 19          | 50       | 24      | 43.6     |
| Diabetes                   |              |                 |              |          |          |          |
| No                         | 83          | 89.2            | 36          | 94.7     | 47      | 85.5     |
| Yes                        | 10          | 10.8            | 2           | 5.3      | 8       | 14.5     |
| p16                        |              |                 |              |          |          |          |
| Negative                   | 70          | 75.3            | 34          | 89.5     | 36      | 65.5     |
| Positive                   | 23          | 24.7            | 4           | 10.5     | 19      | 34.5     |
| T stage                    |              |                 |              |          |          |          |
| T1+T2                      | 43          | 46.2            | 15          | 39.5     | 28      | 50.9     |
| T3+T4                      | 50          | 53.8            | 23          | 60.5     | 27      | 49.1     |
| Lymph node metastasis      |              |                 |              |          |          |          |
| Negative                   | 29          | 31.2            | 11          | 28.9     | 18      | 32.7     |
| Positive                   | 64          | 68.8            | 27          | 71.1     | 37      | 67.3     |
| Clinical stage             |              |                 |              |          |          |          |
| I + II                     | 18          | 19.4            | 8           | 21.1     | 10      | 18.2     |
| III + IV                   | 75          | 80.6            | 30          | 78.9     | 45      | 81.8     |
| Maximum tumor diameter (MTD) |            |                 |              |          |          |          |
| <3 cm                      | 35          | 37.6            | 14          | 36.8     | 21      | 38.2     |
| ≥3 cm                      | 58          | 62.4            | 24          | 63.2     | 34      | 61.8     |
| Differentiation            |              |                 |              |          |          |          |
| High + High-moderate       | 81          | 87.1            | 35          | 92.1     | 46      | 83.6     |
| Moderate + moderate-low    | 12          | 12.9            | 3           | 7.9      | 9       | 16.4     |

*p value was tested from Chi-square test.
†p value was tested from Fisher’s exact test.
the sensitivity and specificity were 79.07% (0.6479–0.8858) and 61.54% (0.4590–0.7511), respectively (Figure 3D).

Collectively, plasma EVs miR-99a-5p levels distinguished early HNSCC and were associated with HPV infection status of patients with HNSCC.

DISCUSSION

Plasma EVs present several advantages in carrying cargo from tumor tissue that make them reliable and specific for use as diagnostic tools [5]. These include being stable molecules that can be easily detected in peripheral circulation plasma and the fact that their expression has been correlated with clinical-pathological features, thus offering promise as prognostic and predictive biomarkers [21]. We previously demonstrated that plasma EVs TGFβ1 is higher in HNSCC patients than in control patients, and is associated with clinical-pathological features of HNSCC patients. More importantly, plasma EVs TGFβ1 expression could clearly distinguish between HNSCC and control patients with higher diagnostic efficiency than total TGFβ1 expression in plasma [16].

Several studies have reported that plasma EVs miRNAs are potential biomarkers for the diagnosis and survival prediction of HNSCC patients [22]. A recent study found that increased plasma EVs miR-491-5p levels were associated with poor overall survival and disease-free survival in HNSCC patients. Plasma EVs miR-491-5p was able to distinguish HNSCC patients with sensitivity and specificity of 46.6% and 100%, respectively [23].

In the current study, we first confirmed the high expression of miR-99a-5p in para-tumor tissue via miRNA sequencing and further verified it using TCGA data. The upregulation of miR-99a-5p in para-tumor tissue is also consistent with previous studies [19, 24]. Notably, the expression of miR-1246 was consistent in tumor tissue and plasma EVs in our previous study [17]. However, we found that miR-99a-5p expression in tumor tissue and plasma EVs showed an opposite trend in HNSCC patients. This unexpected opposite trend of miR-99a-5p in tissue and plasma EVs was reported by Garrido-Cano [21] and Torres [25] in breast cancer and endometrioid endometrial carcinoma. Moreover, other investigators have demonstrated that several other miRNAs have opposite expression patterns in tissues and peripheral blood [26, 27, 28]. These suggests that circulating miR-99a-5p, especially in plasma EVs, may be a potential biomarker for the detection of HNSCC.

MiR-99a-5p has been reported as a tumor suppressor in cancer. Tamai et al. showed that miR-99a-5p induced cellular senescence in gemcitabine-resistant bladder cancer cells by targeting SMARCD1 [11]. Sun et al. found that miRNA-99a-5p suppresses cell proliferation, migration, and invasion by targeting isoprenylcysteine carboxymethyltransferase (ICMT) in oral squamous cell carcinoma [13]. Based on these findings and our data, we speculated that HNSCC cells might release the tumor suppressor miR-99a-5p into the peripheral circulation through EVs to maintain their survival. Selective EVs packaging and the release of miRNAs have been reported to occur in cancer cells as a means of eliminating tumor suppressors [29, 30, 31]. However, the processes by which miRNAs are selectively packed in plasma EVs remain poorly understood [32, 33]. Our results reinforce the concept of selective release, which may explain the discrepancy between tissue and plasma EVs.

Further, in this study, we observed a relationship between the miR-99a-5p expression level and HPV infection status. Overall, HPV positive was associated with more favorable clinical outcomes in HNSCC patients [2]. Although the miR-99a-5p expression level was not associated with clinical outcomes, patients with high miR-99a-5p expression in tissue had a significantly more p16 positive status in pathological samples than patients with low miR-99a-5p expression. By contrast, patients with high miR-99a-5p expression in plasma EVs had less p16 positive status in pathological samples than that of patients with low expression, which is consistent with the opposite trend we observed in tissue and plasma EVs. Further research is needed to clarify the relationship between miR-99a-5p and HPV infection status.

There were several limitations to this investigation. First, validating the diagnostic efficiency of plasma EVs miR-99a-5p with multi-centric validation research is required. Our study lacked prognostic data, as all cases were newly diagnosed (from October 2019 to October 2021). Therefore, the prognostic value of plasma EVs miR-99a-5p could not be evaluated in this study. Second, although we ruled out the possibility that plasma EVs are derived from platelets and immune cells, whether they are completely derived from HNSCC remains to be discussed. This is also one of the bottlenecks restricting plasma EVs as an indicator of tumor diagnosis and prognosis, which needs more efforts to solve. Third, the reasons for the opposite trend of miRNA in HNSCC tumor tissues and plasma EVs have not been studied in detail. More studies should focus on the key pathways and provide an explanation for the opposite trend.

To the best of our knowledge, this is the first study to evaluate the clinical application of plasma EVs miR-99a-5p as a diagnostic biomarker in HNSCC patients. Our study demonstrates two important clinical findings. First, we found that miR-99a-5p is downregulated in HNSCC tissue and is associated with HPV infection status. Second, we identified the dynamic change of miR-99a-5p between tissue and plasma EVs, and confirmed that plasma EVs miR-99a-5p is a potential diagnostic biomarker for early HNSCC patients.

Collectively, we primarily evaluated the value and provided evidence of plasma EVs miR-99a-5p as a biomarker for early HNSCC. This may help improve early HNSCC detection.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request.
ETHICS STATEMENT
The studies involving human participants were reviewed and approved by Ethics Committee of the Eye & ENT Hospital of Fudan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS
Conceptualization: QH, LL, C-PW, and C-YH; Methodology: QH, Y-JS, and C-YH; Validation and formal analysis: QH, Y-JS, and C-YH; Investigation: QH, Y-JS, and C-YH; Data curation: QH, Y-FZ, X-HY, Y-JZ, and J-YL; Writing—original draft preparation: QH, Y-JS, and C-YH; Writing—review and editing: QH, LL, C-PW, and C-YH; Visualization: QH, Y-JS, and C-YH; Funding acquisition: CYH. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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