Targeting exosomal miR-488 inhibits head and neck squamous cell carcinoma growth by mediating RAB25

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Abstract
Background: Cancer cell-derived exosomes and its packaged miRNAs have been identified to regulate tumor growth and progression. However, its role in head and neck squamous cell carcinoma (HNSCC) and the potential mechanism still need to be further investigated.

Methods: RNA sequencing was conducted to select the dysregulated genes in HNSCC. Gene ontology (GO) analysis and TCGA database were performed to analyze the potential candidate genes for HNSCC progression. Cell viability was analyzed using MTT, and colony formation was visualized using crystal violet staining. Luciferase reporter assay was employed to identify the interaction between miR-488 and RAB25. The role of miR-488 and RAB25 in tumor growth and drug response were investigated in vivo and in vitro.

Results: The dysregulated genes in HNSCC captured the signaling of exosomes biogenesis dysfunction. Compared with the normal cells NP69, HNSCC cells had enriched exosomes and its packaged miRNAs, including miR-488. Luciferase reporter assay showed that RAB25 is a downstream target of miR-488. RAB25 was downregulated in HNSCC patients and predicted a poor prognosis. MiR-488/RAB25 signaling controlled cancer cell viability and colony formation ability in vitro and growth in vivo. Importantly, targeting miR-488 significantly inhibited tumor growth and promoted drug response to chemotherapy, suggesting a potential therapeutic promising for HNSCC. Conclusion These findings demonstrate a tumor-cell derived exosomal miR-488 promotes tumor growth by targeting RAB25 that could be targeted for HNSCC treatment.

Introduction
Head and neck squamous cell carcinoma (HNSCC), including the squamous cell carcinoma of oral cavity, lip, larynx, oropharynx, and hypopharynx, is a major frequently lethal malignancy with a yearly mortality of 40-50% (1, 2). HNSCC is associated with the prognostic factors of tobacco, alcohol consumption, and human papillomavirus (HPV) infection. Furthermore, the survival is longer in patients experienced with HPV-infected cancers (Low grade tumors) rather than patients associated with smoking and alcohol, namely, high-grade tumors exhibit more progression than their low-grade counterparts (3). Patients with HNSCC are commonly diagnosed at a progressed stage, and with
unfavorable prognosis. Despite advances on therapies, such as radiotherapy, chemotherapy, immunotherapy and their combination, the prognosis of HNSCC is dismal once recurrence or metastasis occurs, and the survival time has not been greatly improved (4, 5). Therefore, to explore the potential mechanism of HNSCC prognosis, and find the relevant biomarkers that response to the tumor progression and poor outcomes would be greatly imperative.

Intracellular communication is essential for functional integrity of multicellular organisms. The past few years have focused on the exploration of shed vesicles, on account of that the vesicles not only deposit paracrine information, but also alter the recipient behaviors. Exosomes, a kind of extracellular vesicles that formed by the inward budding or shedding from parent cells and transferred various regulatory elements to the recipient cells, has recently captured attention in cellular communication study (6). Exosomes are nano-sized vesicles with the diameter of 20–200 nm, and carried amount of cellular components, such as RNA, DNA, protein and lipids (7). Recently, exosomes are also found in biological fluids, such as plasma, blood, saliva, urine, as well as cerebrospinal fluid (8). It has been demonstrated that exosomes mediate the crosstalk between cells, and contribute to the interaction with the cellular microenvironment and function reprogramming by carrying biological contents from donor to target cells under both physiological and pathological conditions (9, 10).

Growing evidence shows that exosomes are released by many different cell types, such as immune cells, epithelial cells, and tumor cells (11). Tumor-derived exosomes are now believed to promote tumor progression by inducing cell migration and activating the proliferative and angiogenic pathways. Prior studies on tumor-derived exosomes were reported in several malignancies, such as ovarian cancer and prostate cancer, and were proven to accelerate tumor metastasis (12, 13). Beyond that, HNSCC-derived exosomes were identified to mediate cancer initiation, invasion, and progression (14). Therefore, demarcating the potential mechanism of exosomes that influence the tumor progression is critical.

microRNAs (miRNAs) are a set of small non-coding RNA with approximately 22 nucleotides, and manifest biological function by binding to the 3’-untranslated regions (3’-UTR) of target messenger RNAs (mRNAs). According to the preliminary studies, exosomes can facilitate cancer function by
releasing miRNAs (15, 16). Exosomal miRNAs are suggested to modulate the behaviors of recipient cells by coordinating gene expression (17). To date, owing to the presence of exosomes with high stability of miRNAs, multiple exosome-derived miRNAs have been identified as the biomarkers for diseases diagnosis (18). Specifically, miR-488 is up-regulated in the HNSCC based on the TCGA database (19). In this study, we found that miR-488 is increased in the tumor-derived exosomes of HNSCC. While the tumor growth-related RAB25 was decreased in both clinical tissues and cell lines of HNSCC. Therefore, we further investigated the potential role of exosomal miR-488 on tumor behavior and identified tumor-derived exosomes-enriched miR-488 induced tumor progression by targeting RAB25, and these findings were confirmed by both in vivo and in vitro experiments. Thus, the present work provides promising approaches for further treatment of HNSCC.

Materials And Methods

Public data collection and analysis
A TCGA dataset containing gene expression profiles and HNSCC patients’ survival probability was leveraged from https://tcga-data.nci.nih.gov/tcga/. All those HNSCC cohort include 130 patients with high RAB25 expression and 389 patients with low RAB25 expression, which was divided based on a criteria with a false discovery rate (FDR) adjusted p < 0.01, the log(fold-change) > 2.0 defined as downregulated, while the log(fold-change) < −2.0 was defined as upregulated, respectively. The survival endpoints of all patients were retrieved.

Patients And Tissues Collection
A total of 31 patients with HNSCC enrolled in the present study were first diagnosed at the First Affiliated Hospital of Zhengzhou University on the basis of histopathological evaluation, and the clinicopathological and follow-up information were detailed recorded. None of the patients received preoperative radiotherapy, chemotherapy, or immunotherapy before the surgery. The tumor tissues and the paired-adjacent normal tissues were collected underwent the surgery. All tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until required. All procedures performed in this study were in accordance with the ethical standards of the Ethics Committee of the First Affiliated Hospital of Zhengzhou University and with the 2008 Helsinki declaration. The written informed consent was obtained from all participants prior to the samples
collected.

**Real-time Pcr (qrt-pcr)**

To quantify RAB25, ALIX, and CD63 expression, total RNA was extracted from cells or tissues using TRizol reagent (Invitrogen), and NanoDrop-1000 spectrophotometer was used to determine RNA concentration. The isolated RNA was reverse transcribed into cDNA using high-capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR using SYBR Green I technology was then performed based on the ABI Prism 7500 System (Life Technologies) following the manufacturer's instructions.

For miRNA expression, exosomal miRNAs were isolated by using the SeraMir Exosome RNA Purification Kit (System Biosciences, Mountain View, USA), and cDNA for miRNAs was synthesized using 1 µg of total RNA treated with DNase I and TaqMan microRNA assay kit (Applied Biosystems, Foster City, USA) as described in the manufacturer's protocol. The qRT-PCR was conducted with the ABI Prism 7500 System using microRNA detection kit (RiboBio, Guangzhou, China).

All experiments were undertaken in triplicate and normalized against β-actin. Relative mRNA and miRNA expression were calculated using $2^{-\Delta\Delta Ct}$ method.

**Microarray And Data Analysis**

For microarrays assay, the tumor tissues from 31 HNSCC patients and the randomly selected 10 paired-adjacent normal tissues were collected underwent uvulopalatopharyngoplasty (UPPP) surgery and prepared as described previously (20). Total RNA isolated from the tissues was used for mRNA microarray at Beijing Genomics Institute (BGI, Shenzhen, China). Microarray data process and analysis were conducted using Illumina BeadStudio software. To understand the biological functions of those proteins, gene ontology (GO) analysis was performed based on the differentially expressed proteins using DAVID 6.7. A p-value < 0.01 denoted significant enrichments in the GO pathways.

**Cell Culture**

Normal nasopharyngeal epithelial cells NP69, Nasopharyngeal carcinoma cell line CNE-1, Human oral squamous cell carcinoma cell lines HN4, and HN6, and Human Embryonic Kidney (HEK-293) were purchased from Cell Bank of the Chinese Scientific Academy. All cells were routinely grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C.
with 5% CO₂ and 95% air.

**Cell Transfection**

For cell transfection, the mimics and the inhibitors of miR-488, and the negative control, siRNAs and pcDNA of RAB25, and their negative control were purchased from GenePharma (Shanghai, China). The transfection was conducted using Lipofectamine™ 2000 (Thermo Fisher Scientific, US) according to the manufacturer’s instructions. After 24 h, cells were collected for detection of transfection efficiency.

**Western Blot**

For determine protein expression of RAB25, CD63, TSG101, and their negative control GAPDH. Cells were lysed using RIPA buffer, and after quantified by BCA protein assay kit (Beyotime, China), total protein was loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) for electrophoresis and subsequently transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with primary antibody at 4 °C overnight, followed by incubation with peroxidase-linked secondary antibodies (1:10,000) for 1 h. The primary antibodies included in anti-CD63, anti-TSG101, anti-RAB25 and anti-GAPDH, which were all purchased from Abcam. The protein bands were visualized using an enhanced chemiluminescence reagent (Thermo Fisher Scientific, USA). The relative protein expression was normalized to GAPDH.

**Dual-luciferase Reporter Assays**

The miR-488 and RAB25 interaction information was first predicted using TargetScan database. The experimental verification was conducted based on the dual-luciferase reporter assays. Briefly, the sequences of RAB25 3′-UTR (wild-type 3′-UTR) reporter plasmids was amplified. Site-directed mutagenesis (mut) of RAB25 to the miR-488-binding site was also conducted. The wildtype 3′-UTR or mut 3′-UTR was cloned into the psiCHECK-2 vector for luciferase reporter assays. The vector was co-transfected with miR-488 mimics or the control sequence into the HEK-293 cells using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA), and firefly luciferase activity was measured by Reporter Assay System Kit (Promega) 24 h after transfection.

**Cell Colony Formation**

Cells were evenly plated in a 6-well plate at a density of 300 cells per well. After overexpression or
knockdown of RAB25 or miR-488 for 12 h, the medium was refreshed once a day. After 14 days of culture, cells were fixed using 4% Paraformaldehyde for 15 min, and washed with PBS. The colonies were stained with 0.1% Crystal violet staining solution for 30 min and finally calculated using ImageJ software.

Cell Viability
Cells were planted in a 96-well plate at a density of 2000 cell per well for 24 h to allow cell adherence. Cell viability was measured using MTT assays. Briefly, after incubating with 15 μl MTT solution (5 mg/ml) (Sigma-Aldrich, MO, USA) in each well for 4 h, the absorbance was measured under 570 nm wavelengths using a microplate reader. Cell growth curves were depicted using GraphPad Prism 7 software.

Exosome Isolation
Exosomes isolation was conducted using ultrafiltration-ultracentrifugation protocol. After cells reached a confluence of approximately 70-80% in the standard medium, the supernatant without of cells and debris was collected for ultrafiltration at 4000 g, 4 °C for 25 min, followed by ultracentrifugation at 120,000 g for 90 min at 4 °C. The exosome pellet was washed in PBS followed by another ultracentrifugation at 120,000 g for 90 min at 4 °C. The collected exosomes were resuspended in PBS. Western blot analysis, electron microscopy analysis (TEM), and nanoparticle tracking analysis (NTA) were conducted to validate the exosome characterization.

In vivo tumorigenesis
For this study, NOD/SCID mice at 6–8 weeks old were purchased from Shanghai Lab. Animal Research Center (Shanghai, China), and housed in the ventilated cage under condition of 25 °C, with 70% humidified air. All mice were kept at a 12 h light/night circle for one week before the experiment. For xenograft culture, \(1 \times 10^5\) HN4 or HN6 cells that overexpressed miR-488 or its negative control were suspended in the 100 μL PBS and subcutaneously injected into the mice. Tumor volumes were monitored once a week for 4 weeks. In addition, to visualize the role of RAB25 on mice survival, NOD/SCID mice were randomly divided into 10 groups with each group included in 6 mice. The mice were received the subcutaneously injection of HN4 cells with miR-448 knockdown or normal HN4 cells at the number of 1000, 2000, 4000, 8000, and 16000, respectively. Four weeks later, to monitor the
survival mice. To observe the role of miR-488 on mice drug responses, NOD/SCID mice were randomly divided into 4 groups, as follows: mice were intravenously injected with 10 mg/kg Docetaxel (2 mg/mL dissolved in 0.9% saline) every second day for 6 days, or $1 \times 10^5$ HN4 cells pretreated with miR-488 inhibitor, or intravenously injected with 10 mg/kg Docetaxel + miR-488 inhibitor HN4 cells, or intravenously injected saline with the same volume of Docetaxel. Mice survival was visualized and tumor tissues were obtained for further experiments.

The animal experiments were conducted by the approval of the Ethics Committee at the First Affiliated Hospital of Zhengzhou University. The animals use and care were in accordance with the guidelines of this committee.

Statistical analysis
Data were presented as means ± SD. SPSS 18.0 and GraphPad Prism 7 software was conducted to analyze statistics. Unpaired t-test was performed to analyze the differences between two groups, while one-way ANOVA followed by Holm-Oak’s multiple comparison was conducted to compare the difference among more than two groups. A value of $p < 0.05$ was considered significant.

Results
Exosome biogenesis is dysfunction in HNSCC cells
First, we investigated the differentially expressed genes in HNSCC using RNA sequencing and GO cluster analysis. Compared with the para-tumorous tissues, we found 170 genes were dysregulated in tumor tissues, and a cluster analysis based on those differentially expressed genes between the tumor tissues and para-tumorous tissues were generated (Fig. 1a, Fold-change $\geq 2.0$, $p \leq 0.01$). Gene Ontology (GO) analysis showed that those differentially expresses genes were prominent in retinol dehydrogenase activity, extracellular space and especially extracellular exosome (Fig. 1b), thus inferring exosome biogenesis/secrection was dysregulated in HNSCC. Next, we investigated the expressions of exosomal markers ALIX and CD63 in tumor cell lines HN4, CNE1, HN6 and normal cell lines NP69, suggesting a significant elevation of mRNA level of ALIX and CD63 in tumor cells compared with NP69 (Fig. 1c, 1d). More importantly, after comparing the numbers of exosomes in tumor cell lines HN4, CNE1, HN6 and normal cell lines NP69, we found that the exosomes number was dramatically increased in the tumor cell lines (Fig. 1e). Western blot also confirmed that exosome-
related proteins CD63 and TSG101 were enhanced in tumor cells (Fig. 1f). The data indicates that exosome biogenesis is enhanced in HNSCC cells.

**A changed miRNAs expression profile in HNSCC cells-derived exosomes**

Exosomes contain a set of functional nucleic acids such as mRNAs, miRNAs, LncRNAs, and are the main source of circulating miRNAs (21). Next, we performed miRNA microarray to investigate the differentially expressed miRNAs in NH4 compared to NP60. Gene Ontology (GO) analysis showed that several miRNAs were dysregulated in NH4 (Figure 2a). Within it, miR-488 was one of the most aberrant miRNAs with increased level (Figure 2b). Meanwhile, qRT-PCR confirmed that tumor cells exhibited an increased expression of miR-488 compared with NP69 (Figure 2c). To verify the up-regulated miR-488 contributed to dysregulated exosome biogenesis. We performed shRNA specifically targeting CD63 or TSG101 and detected the changes of exosome numbers and miR-488 expression. It showed that knockdown of CD63 (shCD63) or TSG101 (shTSG101) significantly resulted in decreased exosome number and miR-488 level in NH4 and NH6 cells (Figure 2d-2g). The results showed that HNSCC cells-derived exosomes had a changed expression profile of miRNAs including miR-488.

**Rab25 Is A Direct Target Of Mir-488**

Next, to investigate the biological effects of exosome and its contained miR-488 in HNSCC, the potential downstream targets of miR-488 were predicted using online TargetScan. Considering increased level of miR-488 in HNSCC, the potential candidates should be one of the downregulated genes in HNSCC (Fig. 1a). These two searching conditions selected RAB25 as a possible candidate target of miR-488. Subsequently, we further explored the relationship between miR-488 and RAB25 in HEK293 cells using qRT-PCR and Western blot. It exhibited that knockdown of miR-488 enhanced mRNA and protein expression of RAB25 (Fig. 3a and 3c), and overexpression of miR-488 induced decreased expressions of RAB25 (Fig. 3b and 3c). Notably, TargetScan predicted that miR-488 potentially bound to 3’UTR of RAB25 (Fig. 3d). Dual-luciferase reporter assay confirmed that overexpression of miR-488 significantly suppressed the luciferase activity in wild-type of RAB25, but not the mutant one (Fig. 3e). The data indicated that RAB25 is a direct target of miR-488.

**Rab25 Mediates Tumor Cell Viability And Colony Formation**

RAB25, a member of the Rab11 small GTPase family, mediates many cellular processes, and is a
down-regulated gene in oral and oropharyngeal squamous cell carcinoma (22). Previous report revealed that the lower expression of RAB25 is associated with the poor outcome of HNSCC (23). Subsequently, given that RAB25 as a downstream effector of exosome-derived miR-488 may contribute to tumor growth of HNSCC, we investigated the effects of RAB25 on cell viability and colony formation of HNSCC. Interestingly, MTT assays suggested that two different shRNA targeting RAB25, designated as shRAB25-1 and shRAB25-2, predominantly promoted cell viability and colony formation in both HN4 and HN6 cells compared with control (Fig. 4a-4c). Moreover, HNSCC cells with RAB25 overexpression displayed decreased cell viability and colony formation compared with the control cells (Fig. 4d-4f). Based on those data, we concluded that RAB25 mediates cell viability and colony formation of HNSCC.

RAB25 is decreased in patients with HNSCC, and associated with poor prognosis

Next, the expression level of RAB25 in HNSCC tumor tissues was validated by qRT-PCR, and its relationship with clinical feature was analyzed. Consistent with the microarray data (Fig. 1a), qRT-PCR results demonstrated that RAB25 level exhibited a remarkable reduction in tumor tissues and tumor cell lines HN4, HN6 and CNE-1 compared with the normal tissues and cells (Fig. 5a and 5b). We also evaluated the prognosis for RAB25 expression profiles from the patients with HNSCC of online available TCGA data. It demonstrated that low RAB25 expression resulted in a lower survival rate (Fig. 5c). In addition, we found that RAB25 expression was relatively lower in patients with higher node stratification and tumor grades (Fig. 5d-5f). The expression patterns of RAB25 validated its functional role in HNSCC pathogenesis and progression.

Exosomal miR-488 inhibits HNSCC cells proliferation, migration and tumor growth by mediating RAB25

To further validate whether miR-488/RAB25 signaling modulates HNSCC behavior, both HN4 and HN6 cells were overexpressed with miR-488 or RAB25. We found overexpressed miR-488 resulted in an increased cell proliferation and colony formation, while a restrained cell proliferation and colony formation were observed in HN4 and HN6 cells after RAB25 overexpression (Fig. 6a-6d). To determine the role of miR-488 on tumor formation in vivo, miR-488-overexpressed HN4 and HN6 were subcutaneously injected to the mice, respectively, and the results demonstrated that overexpression
of miR-488 increases tumor volume (Fig. 6e and 6f). To investigate its clinical therapeutic effects, we firstly performed the in vivo limiting-diluting assay to explore the effects of miR-488 inhibitor on tumor-initiating ability. Mice were subcutaneously injected with miR-488 inhibitor-treated HN4 cells or their negative control at the number of 1000, 2000, 4000, 8000, and 16000, respectively, and we found that miR-488 inhibitor significantly decreased its tumor-initiating ability in vivo (Fig. 6g), suggesting its potential target for clinical therapy. Next, we investigated the clinical therapeutic efficacy using miR-488 inhibitor and conventional chemical drug docetaxel. Interesting, when combining application, miR-488 inhibitor induced a synergistic effect for docetaxel to inhibit tumor growth in vivo (Fig. 6h), although a limited therapeutic efficacy for miR-488 inhibitor. Those results uncovered a therapeutic role for HNSCC treatment by targeting miR-488.

Discussion
Tumor microenvironment is important for the interaction between tumor cells and stromal cells and mediates the initiation and progression of various cancers. It has been reported that the assembly and release of tumor derived exosomes is modulated by tumor microenvironment (24). On the contrary, the tumor-derived exosomes influence the function of tumor microenvironment by affecting tumor cell behaviors (25). Mounting studies have supposed that the tumor cells can release much more exosomes than that the parental non-tumor cells. Therefore, exosomes have attracted attention for clinic tumor therapy (26). In the present study, we found that HNSCC produced larger number of exosomes, which carrying miR-488 modulating cell viability, colony formation, drug response by targeting RAB25.

Recently, small guanosine triphosphates (GTPases) are emerging as novel regulators on cancer development and progression (27). RAB25 is a small GTPases that belongs to the RAB11 subfamily, which is the member of RAB protein family, and is believed as a tumor suppressor in HNSCC subtypes (23, 27). It has been demonstrated that the expression of RAB25 is associated with tumor progression, and loss of RAB25 leads to an induction of cell migration and invasion (28). In the current study, we found that RAB25 is reduced in HNSCC, and overexpression of RAB25 strongly hampers the tumor cells viability and colony formation. Besides, most dysregulated genes obtained from the
microarray displayed a function of extracellular exosome. Thus, we hypothesized that RAB25 mediated the HNSCC behaviors by controlling exosome function.

Exosome contents have been demonstrated to modulate the function of recipient cells (12). miRNAs in the circulating exosomes can assert cancer diagnosis due to that they facilitate cancer progression by regulating the biological behaviors of proteins (29). Our present study found that miR-488 is significantly increased in the HNSCC cells and tissues. Attractively, the 3’UTR of RAB25 has a binding site with miR-488, who can target RAB325 to negatively regulate its expression. Therefore, we inferred that the aberrant expression of RAB25 is gained by miR-488 to mediate the progression of HNSCC. We documented that tumor-derived exosomes carried abundant of miR-488 in HNSCC cells and exhibited induced cell viability and colony formation ability.

It has been reported that tumor progression is connected with the development of drug resistance (30). Herein, we found that up-regulated RAB25 induced the decreased both cell proliferation and colony formation ability in vitro. In vivo experiments demonstrated that down-regulated RAB25 resulted in a high survival rate. Importantly, HNSCC mice showed chemoresistance to docetaxel when pretreated with miR-488 inhibitor, and the tumor tissues showed decreased stemness, indicating that the increased miR-488 promoted drug resistance and cell stemness by targeting RAB25.

**Conclusions**

In summary, our study identified the crucial role of tumor-derived exosomes and its packaged miRNAs on tumor progression, drug response. Importantly, we elucidated that tumor-derived exosomes enriched miR-488 mediates tumor cell proliferation, colony formation, tumor growth and drug response by targeting its downstream effector RAB25 that have potential therapeutic efficacy for HNSCC treatment.

**List Of Abbreviations**

Head and neck squamous cell carcinoma (HNSCC)

Gene ontology (GO)

Human papillomavirus (HPV)

Messenger RNAs (mRNAs)
MicroRNAs (miRNAs)
3’-untranslated regions (3’-UTR)
Guanosine triphosphates (GTPases)
False discovery rate (FDR)
Uvulopalatopharyngoplasty (UPPP)
Fetal bovine serum (FBS)
Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE)
Mutagenesis (mut)
Electron microscopy analysis (TEM)
Nanoparticle tracking analysis (NTA)

Declarations
Ethics approval and consent to participate
The study was approved by the First Affiliated Hospital of Zhengzhou University.
Consent for publication
This study was undertaken with the consent of the First Affiliated Hospital of Zhengzhou University.
Availability of data and material
Not applicable.
Competing interests
The authors declare that they have no competing interests.
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Authors’ contributions
Xueping Wang conceived the study and drafted the manuscript. Xueping Wang and Xiaoyuan Zhu performed the experiments. Yulin Zhao contributed to the quality control.
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Exosome biogenesis is dysfunction in HNSCC cells (a) Heatmap shows the differentially expressed genes in HNSCC tissues compared with the adjacent tissues. (b) Enrichment pathway analysis was performed to analyze the dysregulated pathways in HNSCC tissues. (c and d) Representative ALIX and CD63 mRNA expression in HNSCC cell lines. (e) Comparison of the exosome number between NP69 and HNSCC cell lines. (f) Western blot was conducted to detect extracellular vesicle-related protein marker CD63 and exosome-specific marker TSG101 in HNSCC cell lines. *p<0.05, **p<0.01.
Figure 2

A changed miRNAs expression profile in HNSCC cells-derived exosomes (a) volcano plot shows the dysregulated miRNAs in tumor-derived exosomes. (b) the expressions of representative dysregulated miRNAs in exosomes were confirmed by qRT-PCR. (c) the expressions of miR-488 expression in the HNSCC cell lines and normal NP69 cells. (d, e) Exosome numbers in both NH4 and NH6 cells with CD63 or TSG101 knockdown or negative control ones. (f, g) Relative miR-488 expression in both NH4 and NH6 cells with CD63 or TSG101 knockdown or negative control ones. *p<0.05, **p<0.01.
RAB25 is a direct target of miR-488. The mRNA and protein expression of RAB25 in HEK293 cells with miR-488 knockdown (a, c) or miR-488 overexpression (b, c). (d) Graphic illustration showed the miR-488 binding site of RAB25 3’UTR. (e) Luciferase activity in HEK293 cells with WT or mutated miR-488 binding site. **p<0.01, ***p<0.001.
RAB25 mediates tumor cell viability and colony formation. (a, b) MTT assay was performed to detect cell viability of HN4 and HN6 with RAB25 knockdown. (c) Cell colony number was visualized in HN4 and HN6 cells with RAB25 knockdown of RAB25. (d, e) MTT assay was performed to detect cell viability of HN4 and HN6 with RAB25 overexpression. (f) Cell colony number was visualized in HN4 and HN6 cells with RAB25 overexpression. **p<0.01, ***p<0.001.
RAB25 is decreased in patients with HNSCC, and associated with poor prognosis (a) mRNA expression of RAB25 in HNSCC tumor and adjacent tissues. (b) mRNA expression of RAB25 in HNSCC cell lines and normal cells. (c) Survival time of HNSCC patients with high or low RAB25 expression based on TCGA data. (d-f) transcriptional level of RAB25 in HNSCC and healthy tissue (d) or different clinical node (e) or grade (f). ***p<0.001.
Exosomal miR-488 inhibits HNSCC cells proliferation, migration and tumor growth by mediating RAB25. (a, b) Cell proliferation was detected in HN4 and HN6 cells with miR-488 overexpression or miR-488 and RAB25 co-overexpression. (c, d) Cell colony formation was detected in HN4 and HN6 cells with miR-488 overexpression or miR-488 and RAB25 co-overexpression. (e, f) Tumor volume was determined in mice injected with miR-488-overexpressed HN4 or HN6 cells or negative control cells. (g) tumor initiation was monitored in mice that were subcutaneously received different numbers (1000, 2000, 4000, 8000 and 16000) of miR-488 inhibitor-treated HN4 cells or their negative control cells. (h) the tumor size (drug response) were monitored in tumor-bearing mice that were received miR-488 inhibitor or docetaxel or both. *p<0.05, **p<0.01, ***p<0.001.