Analysis of functional-RNA network starting from exosomal microRNAs in oral squamous cell carcinoma cells

Tadashi Masaoka

Nihon University Graduate School of Dentistry,

Major in Oral and Maxillofacial Surgery

（Directors: Prof. Morio Tonogi and Assist. Prof. Keiji Shinozuka）

Index

This doctoral thesis was prepared using the original article “Bioinformatics analysis of dysregulated exosomal microRNAs derived from oral squamous cell carcinoma cells” (Journal of Oral Science, 2021 *in press*) with new unpublished data (Figure 4).

Abstract

Secreted membrane vesicles called exosomes play important roles in intercellular communication, transferring information by delivering biological contents that modulate the functions of recipient cells. Exosomes contain various molecules, such as proteins, microRNAs (miRNAs), and mRNAs, and are found in cell culture supernatants and almost all human biological fluids. Oral squamous cell carcinoma (OSCC)-derived exosomal miRNAs may be used as diagnostic and therapeutic biomarkers. However, a systematic analysis of OSCC exosomal miRNAs has not been reported. Therefore, this study was aimed to identify exosomal miRNAs associated with OSCC and discover their functions and clinical significance. Exosomal miRNAs that were differentially expressed in four OSCC-derived cell lines (HSC-2, HSC-3, Ca9-22, and Ho-1-N1) compared with those in human normal keratinocytes were examined using microarray analysis, and 20 miRNAs were obtained. To identify important functional networks and gene ontologies in OSCC, the identified miRNAs and their potential target genes were analyzed using Ingenuity Pathway Analysis. Six highly significant genetic networks and four highly significant upstream miRNAs (miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p) which were associated with tumorigenesis, tumor development, and regulation of the epithelial-mesenchymal transition were detected. In particular, miR-125b was a central node in the most highly significant network. Gene ontology analysis showed significant enrichment of genes with cancer-related functions. Downstream effector analysis revealed enrichment in functional categories related to cell death and apoptosis, while functional category related to cancer. In canonical pathway

analysis, predicted target genes of the dysregulated miRNAs were enriched in the molecular mechanisms of cancer pathway and cell cycle: G1/S checkpoint regulation pathway. These analyses provide a comprehensive view of the functions of dysregulated exosomal miRNAs in OSCC, providing insight on its tumorigenesis and development. Additionally, these four miRNAs may crucial roles in OSCC and represent molecular targets for diagnostic biomarkers.

Introduction

Despite progress in the understanding and treatment of oral cancer, it remains the sixth most commonly diagnosed cancer worldwide, causing approximately 145,000 deaths annually [1]. Oral squamous cell carcinoma (OSCC) accounts for 90% of all oral cancer cases [2]. Tobacco and alcohol consumption are the main risk factors for the disease and human papilloma virus is also related to a subset of cases [3]. Current therapeutic options for OSCC are surgery, chemotherapy, radiotherapy, and immunotherapy. However, despite diagnostic and therapeutic advances, the 5-year overall survival rate of OSCC has remained unchanged for decades (at approximately 50-60%), and the disease also has a high metastatic potential [4]. In addition, traditional cancer-screening techniques, such as detecting and imaging protein biomarkers, are not sufficient for early OSCC detection and diagnosis [2]. Therefore, an enhanced understanding of the molecular mechanisms underlying OSCC tumorigenesis is crucial to ensure early diagnosis, improve prognosis prediction, and establish effective therapies.

Cells communicate with neighboring and distant cells via secretion of extracellular vesicles (EVs) [5]. Exosomes, first discovered in 1983, are 50-150 nm in diameter and exist in the extracellular space and in blood, urine, and saliva [5]. They carry functional molecules, including proteins, metabolites, lipid mediators, and nucleic acids (mRNAs, non-coding RNAs, and DNA) [5]. Exosomes are involved in important processes such as immune responses and tissue repair [6]. However, tumor cells can also secrete large amounts of exosomes, which alter their environment and enable them to grow and disseminate by triggering vascular permeability and conditioning premetastatic sites [6]. Therefore, exosomes have potential clinical applications

as they may contain biomarkers and therapeutic targets, and their utility in OSCC diagnosis and treatment is receiving considerable attention.

The presence of mRNAs and microRNAs (miRNAs) in exosomes was first reported in 2007 [7]. The profile of exosomal miRNAs (which are 22-25 nucleotides long) is similar to that of cellular miRNAs. These molecules play roles in stem cell differentiation, hematopoiesis, exocytosis, differentiation, organogenesis, and tumorigenesis [7]. The specificity and distinct signatures of tumor exosomal miRNAs, as well as their involvement in tumorigenesis, invasion, angiogenesis, progression, metastasis, and chemoresistance, have been reported [7]. Fukumoto et al. reported that in OSCC tumors, miRNA-26a and -26b enhance cell migration and invasion by regulating the expression of transmembrane protein 184B [8]. Kawakubo et al. demonstrated that exosomal miR-200c-3p induces an invasive phenotype in previously non-invasive cells within the OSCC tumor mass [9]. Thus, exosomal miRNAs have potential for both diagnostic and therapeutic applications. Since exosomes can be isolated using minimally invasive methods, their use as novel molecular diagnostic tools in cancer therapy has recently sparked interest in the use of liquid biopsies. Exosomes may provide more sensitive biomarkers for cancer detection, as exosomal miRNAs have greater stability than cellular miRNAs [9]. Several studies have reported clinical applications of liquid biopsies, including assessment of exosomal miRNAs in head and neck squamous cell carcinoma (HNSCC). Lin et al. reported increased expression of miR-21 and miR-24 in HNSCC [10], whereas Summerer et al. suggested that high levels of miR-142, miR-186, miR-195, miR-374b, and miR-574 are prognostic biomarkers for the disease [11].

Although several exosomal miRNAs have been reported as potential biomarkers and their biological functions have been revealed, a systematic analysis of OSCC exosomal miRNAs has not been reported. The aim of this study was to identify exosomal miRNAs associated with OSCC and discover their functions and clinical significance.

Materials and Methods

Cell culture

Human normal oral keratinocytes (HNOKs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA, No. 2610) and cultured in an oral keratinocyte medium (ScienCell Research Laboratories) according to the manufacturer's instructions. The OSCC-derived cell lines HSC-2 (JCRB0622), HSC-3 (JCRB0623), Ca9-22 (JCRB0625), and Ho-1-N1 (JCRB0831) were obtained from the Human Science Research Resources Bank (Osaka, Japan). All the cell lines were tested and declared free of mycoplasma infection by the company and used for experimentation within three months after purchase. All OSCC-derived cell lines were maintained in Dulbecco's modified Eagle's medium/F-12 HAM (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Co.) and 50 U/mL of penicillin-streptomycin (Sigma-Aldrich Co.). All cell lines were cultured at 37°C in a humidified incubator in presence of 5% CO_2.

Isolation of exosomal miRNA from culture media

Cells were cultured without FBS or penicillin-streptomycin for 48 h and the culture media was collected. Each sample of medium (10 mL) was centrifuged for 5 min at 3,200 × *g* to remove the cell debris, and the supernatant was used for exosome isolation. Exosomes were isolated using column purification and the miRCURY Exosome Isolation Kit (Exiqon, Woburn, MA, USA), which recovered all RNA species including miRNAs, following the manufacturer's instructions. The quality and quantity of the extracted RNA were determined using a NanoDrop

ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Samples with optical density $(OD)_{260}/OD_{280}$ ratios of 1.60-2.20 were used in microarray experiments.

MiRNA expression profiling

MiRNA expression profiling was performed on 10 samples (HNOKs n = 2; the OSCC-derived cell lines, HSC-2, HSC-3, Ca9-22 and HO-1-N1: n = 2 each). The sixth-generation miRCURY LNA miRNA polymerase chain reaction (PCR) system (Exiqon) was used containing miRNome human panels I + II (V.4, Exiqon), which consisted of primer sets against 752 established human miRNAs, following the manufacturer's recommended protocol. Briefly, first, miRNAs were reverse-transcribed from total RNA (200 ng) using miRNA-specific reverse transcription primers. The cells produced much lower yields, with RNA concentrations of approximately 50 ng/μL each. Next, the reverse-transcribed miRNAs were amplified using a locked nucleic acid-enhanced PCR primer anchored in the miRNA sequence and a universal PCR primer [12]. The raw data were received as normalized miRNA expression profiles. To identify dysregulated miRNAs, miRNAs that were differentially expressed between OSCC-derived cell lines and HNOKs by ≥ 1.5-fold were analyzed.

MiRNA target gene prediction and gene ontology analysis

Ingenuity Pathway Analysis (IPA, QIAGEN Inc., St. Marshall, CA, USA, https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis) was used to identify the biological functions of differentially expressed miRNAs and the signaling pathways

involving their potential targets [13-15]. Genes were categorized based on their location, cellular components, and reported or suggested molecular functions. The identified genes were also mapped to genetic networks available in the IPA database, and a score based on the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone was obtained. A score of 3 indicated a 1/1,000 chance that a gene was in a network by chance (i.e., a 99.9% confidence level); therefore, this score was used as a threshold when identifying gene networks. The causal analytics tools 'Gene ontology analysis', 'Upstream regulator analysis', 'Downstream effector analysis', and 'Canonical pathway analysis' are implemented and available within IPA. For downstream effector analysis, the Z-score > 2 was defined as the threshold of significant activation, whilst Z-score < -2 was defined as the threshold of significant inhibition. For the canonical pathway analysis, the -log (*P*-value) > 2 was taken as threshold. *P*-value was calculated automatically by IPA using right-tailed Fisher's exact test. The algorism used for calculating the Z-scores and *P*-value has been described previously [15].

Results

Exosomal miRNA expression in OSCC-derived cell lines

To identify differentially expressed exosomal miRNAs between OSCC-derived cell lines and HNOKs, miRNA array expression profiling was performed. Compared with HNOK-derived exosomes, HSC2, HSC3, Ca9-22, and Ho-1-N1 cell-derived exosomes contained 183, 129, 179, and 139 upregulated (Fig. 1A) and 111, 82, 136, and 99 downregulated (Fig. 1B) miRNAs, respectively. Eight upregulated and 12 downregulated miRNAs were identified in all four OSCC-derived cell lines compared with those in HNOKs.

Genetic network and ontology analysis

First, the IPA miR target filter was utilized to identify potential mRNA targets of the 20 differentially expressed miRNAs. The analysis revealed 236 potential mRNA targets based on experimental evidence. Next, genetic network analysis of the regulated miRNAs and their target genes was performed using IPA. These networks indicated functional relationships between gene products based on published interactions. Six highly significant networks were observed with some common biological functions, including cellular development, cellular growth and proliferation, and cell death and survival (Table 1). These networks were significant in OSCC (i.e., they were composed of more identified genes than would be expected by chance). The network with the highest score (network 1) was centered around miR-125b-5p (Fig. 2), which was linked to the majority of the genes in the network. To strengthen the gene regulatory network data and gain deeper insights regarding the functions of target genes and their regulation,

the relationships of the top four upstream miRNAs (miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p) that were highly relevant according to IPA upstream regulator analysis were combined into an integrated target gene regulatory network (Table 2). MiRNA expression profiling results of the top four upstream miRNAs were shown in Table 2. Gene ontology analysis was also performed using the IPA tool. Cancer was the most significantly enriched functional category (Table 3). Enriched biological processes in the integrated network (Fig. 3) included advanced malignant tumor, epithelial-mesenchymal transition (EMT), and primary solid tumor, and contained several important cancer-related targets. The associated downstream targets included transforming growth factor beta receptor 2 (TGFBR2), vascular endothelial growth factor A (VEGFA), vimentin (VIM), E2F transcription factor 1 (E2F1), RB transcriptional corepressor 1 (RB1), BCL2 apoptosis regulator (BCL2), signal transducer and activator of transcription 3 (STAT3), tumor necrosis factor, and zinc finger and BTB domain-containing 7A (ZBTB7A). The upregulated targets included phosphatase and tensin homolog (PTEN), zinc finger protein, FOG family member 2 (ZFPM2), E2F transcription factor 3 (E2F3), stearoyl-CoA desaturase, erb-b2 receptor tyrosine kinase 3 (ERBB3), and tumor protein p53 (TP53). Furthermore, downstream effector analysis provides deeper insight into the contribution of the 20 differentially expressed exosomal miRNAs and identified potential mRNA targets of these miRNAs into cellular and functional processes. Fig. 4A presents a high-level tree map of affected downstream functional categories in OSCC-derived cell lines compared to HNOKs based on miRNA expression profiling data. Remarkably, functional categories related to cancer were under presented in OSCC (Fig. 4B). In contract, enrichment in functional categories

related to cell death and apoptosis was observed in OSCC (Fig. 4C).

Canonical pathway analysis

To further understand the relationships between miRNAs and their target mRNAs, the target genes were categorized according to signaling pathways using the IPA tool. The top 30 canonical pathways are listed in Fig. 5. Several pathways related to cancer were observed. Among these pathways, molecular mechanisms of cancer were the most significant, followed by cell cycle: G1/S checkpoint regulation. Network analysis also identified cell cycle regulation as a crucial cancer-related function of OSCC exosomes.

Discussion

In this study, the potential regulatory effects of dysregulated exosomal miRNAs released by OSCC-derived cell lines were assessed. 20 (eight up- and 12 downregulated) exosomal miRNAs that were differentially expressed in all four OSCC-derived cell lines were identified compared to HNOKs using miRNA microarray analysis. After the analysis of the 20 miRNAs and their potential corresponding target genes, six genetic networks (Table 1) including four miRNAs (miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p) that were significantly associated with cancer were detected. In particular, miR-125b was a central node in the network of highest significance; therefore, its related network was used for pathway construction. The enriched canonical pathways confirmed the relationships between the identified exosomal miRNA networks and cancer. Furthermore, downstream effector analysis revealed enrichment in functional categories related to cell death and apoptosis, while processes related to cancer were suppressed. It was speculated that these four miRNAs might play crucial roles in OSCC, and further investigations for their biological activities would contribute to understanding of tumorigenesis and tumor development.

MiR-125b is an established oncogenic miRNA. It is upregulated in various cancers [16-18] and its downregulation inactivates the tumor suppressor function of TP53 in human lung fibroblasts [19] and oral cancer [20]. MiR-125b is significantly downregulated in tongue squamous cell carcinoma compared with that in normal adjacent tissue, and this result matched with the patterns observed for other oral cavity carcinomas [20]. In a different study, Henson et al. reported that miR-125b downregulation is important for OSCC development and progression

[21]. Furthermore, in a previous study, downregulation of miR-125b was observed in OSCC-derived cell lines [22]. MiR-125b-transfected cells showed a decreased proliferation rate and enhanced radiosensitivity to X-ray irradiation [22]. Moreover, miR-125b expression correlated with OSCC tumor staging and survival. These data indicate that dysregulating miR-125b expression or activity might contribute to tumorigenesis by promoting OSCC proliferation [22].

MiR-17 binds to the 3′ untranslated region of integrin β8 to suppress its translation and is a potential prognostic marker for OSCC outcome and metastasis [23]. Ping et al. revealed that miR-17-5p promotes the proliferation and migration of human tongue squamous cell carcinoma cells and inhibits autophagy under hypoxia [24]. In addition, miR-17-5p has been linked to the development of many other cancers, including those of colorectum [25], prostate [26], and lungs [27], and its expression level is associated with cancer aggressiveness and therapy resistance [28]. In glioma cells, overexpression of miR-17-5p decreases beclin 1-mediated autophagy [29].

MiR-200b is an important regulator of EMT [30]. Its expression and effects identified in the present study were consistent with a previous report in which miR-200b was shown to be significantly upregulated in patients with World Health Organization (WHO) grade II/III OSCC compared with that in patients with WHO grade I OSCC [31]. In human tongue cancer cells, miR-200b regulates chemotherapy-induced EMT by targeting the BMI1 proto-oncogene, polycomb ring finger [32]. In head and neck cancer, miR-200b overexpression is associated with progression and poor prognosis [31,33].

A role for miR-23a has been demonstrated in prostate cancer [34]. In OSCC, Chen et al.

revealed that miR-23a suppresses tumor proliferation and invasion and promotes apoptosis [35]. Using bioinformatic analyses, it was predicted that these effects were exerted via targeting fibroblast growth factor 2. Moreover, miR-23a is overexpressed in several tumors and promotes invasion and metastasis [36-38].

These studies indicate that miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p function as oncogenes. Therefore, these four miRNAs could represent effective diagnostic markers and/or therapeutic targets for OSCC.

Uptake of tumor exosome miRNAs affects premetastatic or progressive organ stromal cells, producing a tumor-supportive microenvironment [39]. The comprehensive miRNA/target gene expression profiling-assisted pathway analysis is an appealing approach to identify candidate genes and pathways involved in the carcinogenesis of OSCC and other cancers. Four dysregulated miRNAs, miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p, were identified, which are associated with tumor development, EMT regulation, and tumorigenesis. The identification of novel molecular pathways and targets regulated by these miRNAs may lead to a better understanding of OSCC and its tumorigenesis, and the development of new therapeutic strategies for its treatment. Wang et al. reported that miR-23a can be used as a therapeutic tool in cancer management [40]. While these predictions require experimental validation in future studies, they provide new areas for future research focusing on the potential of miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p as prognostic biomarkers and therapeutic targets in OSCC.

Conclusions

This is the first study to use bioinformatic analysis to investigate differences in exosomal miRNAs in the culture supernatants of OSCC cell lines and HNOKs. 4 upstream miRNAs, i.e., miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p were identified. This study provides a comprehensive view of the functions of dysregulated exosomal miRNAs in OSCC, providing insight on its tumorigenesis and development. Additionally, these four miRNAs may crucial roles in OSCC and represent molecular targets for diagnostic biomarkers.

Acknowledgments

I would like to express my sincere appreciation to Prof. Morio Tonogi and Assist. Prof. Keiji Shinozuka for their kind instruction in conducting this study.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. (2015) Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136, E359-E386.
2. Petruzzi MN, Cherubini K, Salum FG, de Figueiredo MA (2017) Role of tumour-associated macrophages in oral squamous cells carcinoma progression: an update on current knowledge. Diagn Pathol, doi:10.1186/s13000-017-0623-6.
3. Farsi NJ, Rousseau MC, Schlecht N, Castonguay G, Allison P, Nguyen-Tan PF, et al. (2017) Aetiological heterogeneity of head and neck squamous cell carcinomas: the role of human papillomavirus infections, smoking, and alcohol. Carcinogenesis 38, 1188-1195.
4. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A (2018) Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 68, 394-424.
5. Tkach M, Théry C (2016) Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. Cell 164, 1226-1232.
6. Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic MM, et al. (2015) Tumour exosome integrins determine organotropic metastasis. Nature 527, 329-335.
7. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9, 654-659.
8. Fukumoto I, Hanazawa T, Kinoshita T, Kikkawa N, Koshizuka K, Goto Y, et al. (2015) MicroRNA expression signature of oral squamous cell carcinoma: functional role of *microRNA-26a/b* in the modulation of novel cancer pathways. Br J Cancer 112, 891-900.
9. Kawakubo-Yasukochi T, Morioka M, Hazekawa M, Yasukochi A, Nishinakagawa T, Ono K, et al. (2018) miR-200c-3p spreads invasive capacity in human oral squamous cell carcinoma microenvironment. Mol Carcinog 57, 295-302.
10. Lin SC, Liu CJ, Lin JA, Chiang WF, Hung PS, Chang KW (2010) *miR*-24 upregulation in oral carcinoma: Positive association from clinical and *in vitro* analysis. Oral Oncol 46, 204-208.

11. Summerer I, Unger K, Braselmann H, Schuettrumpf L, Maihoefer C, Baumeister P, et al. (2015) Circulating microRNAs as prognostic therapy biomarkers in head and neck cancer patients. Br J Cancer 113, 76-82.
12. Mestdagh P, Hartmann N, Baeriswyl L, Andreasen D, Bernard N, Chen C, et al. (2014) Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. Nat Met 11, 809-815.
13. Fushimi K, Uzawa K, Ishigami T, Yamamoto N, Kawata T, Shibahara T, et al. (2008) Susceptible genes and molecular pathways related to heavy ion irradiation in oral squamous cell carcinoma cells. Radiother Oncol 89, 237-244.
14. Shinozuka K, Uzawa K, Fushimi K, Yamano Y, Shiiba M, Bukawa H, et al. (2009) Downregulation of Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 in Oral Squamous Cell Carcinoma: Correlation with Tumor Progression and Poor Prognosis. Oncology 76, 387-397.
15. Krämer A, Green J, Pollard J Jr, Tugendreich S (2014) Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics 30, 523-530.
16. Karbasy SH, Taheriazam A, Mirghasemi A Sedaghati F, Shakeri M, Yahaghi E, Bahador R (2015) Upregulation of miR-300 and downregulation of miR-125b act as potential predictor biomarkers in progression, metastasis, and poor prognosis of osteosarcoma. Tumour Biol, doi: 10.1007/s13277-015-4000-3.
17. Fujino Y, Takeishi S, Nishida K, Okamoto K, Muguruma N, Kimura T, et al. (2017) Downregulation of microRNA-100/microRNA-125b is associated with lymph node metastasis in early colorectal cancer with submucosal invasion. Cancer Sci 108, 390-397.
18. Kassem NM, Makar WS, Kassem HA, Talima S, Tarek M, Hesham H, et al. (2019) Circulating *miR-34a* and *miR-125b* as Promising non Invasive Biomarkers in Egyptian Locally Advanced Breast Cancer Patients. Asian Pac J Cancer Prev 20, 2749-2755.
19. Le MT, Teh C, Shyh-Chang N, Xie H, Zhou B, Korzh V, et al. (2009) MicroRNA-125b is a novel negative regulator of p53. Genes Dev 23, 862-876.
20. Boldrup L, Coates PJ, Wahlgren M, Laurell G, Nylander K (2012) Subsite-based alterations in miR-21, miR-125b, and miR-203 in squamous cell carcinoma of the oral cavity and correlation to important target proteins. J Carcinog, doi:10.4103/1477-3163.104007.

21. Henson BJ, Bhattacharjee S, O'Dee DM, Feingold E, Gollin SM (2009) Decreased expression of miR-125b and miR-100 in oral cancer cells contributes to malignancy. Genes Chromosom Cancer 48, 569-582.
22. Shiiba M, Shinozuka K, Saito K, Fushimi K, Kasamatsu A, Ogawara K, et al. (2013) MicroRNA-125b regulates proliferation and radioresistance of oral squamous cell carcinoma. Br J Cancer 108, 1817-1821.
23. Chang CC, Yang YJ, Li YJ, Chen ST, Lin BR, Wu TS, et al. (2013) MicroRNA-17/20a functions to inhibit cell migration and can be used a prognostic marker in oral squamous cell carcinoma. Oral Oncol 49, 923-931.
24. Pang F, Liu C, Cui Y, Gong K, Liu G, Bian Y, et al. (2019) miR-17-5p promotes proliferation and migration of CAL-27 human tongue squamous cell carcinoma cells involved in autophagy inhibition under hypoxia. Int J Clin Exp Pathol 12, 2084-2091.
25. Li Y, Lauriola M, Kim D, Francesconi M, D'Uva G, Shibata D, et al. (2016) Adenomatous polyposis coli (APC) regulates miR17-92 cluster through β-catenin pathway in colorectal cancer. Oncogene 35, 4558-4568.
26. Guo J, Mei Y, Li K, Huang X, Yang H (2016) Downregulation of miR-17-92a cluster promotes autophagy induction in response to celastrol treatment in prostate cancer cells. Biochem Biophys Res Commun 478, 804-810.
27. Chatterjee A, Chattopadhyay D, Chakrabarti G (2014) miR-17-5p Downregulation Contributes to Paclitaxel Resistance of Lung Cancer Cells through Altering beclin1 Expression. PLoS One 9, e95716.
28. Bobbili MR, Mader RM, Grillari J, Dellago H (2017) OncomiR-17-5p: alarm signal in cancer? Oncotarget 8, 71206-71222.
29. Hou W, Song L, Zhao Y, Liu Q, Zhang S (2017) Inhibition of Beclin-1-Mediated Autophagy by MicroRNA-17-5p Enhanced the Radiosensitivity of Glioma Cells. Oncol Res 25, 43-53.
30. Jahid S, Sun J, Edwards RA, Dizon D, Panarelli NC, Milsom JW, et al. (2012) *miR-23a* Promotes the Transition from Indolent to Invasive Colorectal Cancer. Cancer Discov 2, 540-553.
31. Arunkumar G, Deva Magendhra Rao AK, Manikandan M, Prasanna Srinivasa Rao H, Subbiah S, Ilangovan R, et al. (2018) Dysregulation of miR-200 family microRNAs and epithelial-mesenchymal transition markers in oral squamous cell carcinoma. Oncol Lett 15, 649-657.
32. Sun G, Cao Y, Wang P, Song H, Bie T, Li M, et al. (2018) miR-200b-3p in plasma is a potential diagnostic biomarker in oral squamous cell carcinoma. Biomarkers 23, 137-141.

33. Sun L, Yao Y, Liu B, Lin Z, Lin L, Yang M, et al. (2012) MiR-200b and miR-15b regulate chemotherapy-induced epithelial-mesenchymal transition in human tongue cancer cells by targeting BMI1. Oncogene 31, 432-445.
34. Patil S, Warnakulasuriya S (2020) Blood-based circulating microRNAs as potential biomarkers for predicting the prognosis of head and neck cancer-a systematic review. Clin Oral Investig 24, 3833-3841.
35. Wen YC, Lee WJ, Tan P, Yang SF, Hsiao M, Lee LM, et al. (2015) By inhibiting snail signaling and miR-23a-3p, osthole suppresses the EMT-mediated metastatic ability in prostate cancer. Oncotarget 6, 21120-21136.
36. Chen F, Qi S, Zhang X, Wu J, Yang X, Wang R (2019) miR-23a-3p suppresses cell proliferation in oral squamous cell carcinomas by targeting FGF2 and correlates with a better prognosis: miR-23a-3p inhibits OSCC growth by targeting FGF2. Pathol Res Pract 215, 660-667.
37. Diao H, Ye Z, Qin R (2018) miR-23a acts as an oncogene in pancreatic carcinoma by targeting FOXP2. J Investig Med 66, 676-683.
38. An J, Pan Y, Yan Z, Li W, Cui J, Yuan J, et al. (2013) MiR-23a in Amplified 19p13.13 Loci Targets Metallothionein 2A and Promotes Growth in Gastric Cancer Cells. J Cell Biochem 114, 2160-2169.
39. Rana S, Malinowska K, Zöller M (2013) Exosomal Tumor MicroRNA Modulates Premetastatic Organ Cells. Neoplasia 15, 281-295.
40. Wang N, Tan HY, Feng YG, Zhang C, Chen F, Feng Y (2018) microRNA-23a in Human Cancer: Its Roles, Mechanisms and Therapeutic Relevance. Cancers (Basel), doi: 10.3390/cancers11010007.

Tables

Table 1 Genetic networks in OSCC cell lines

Network	Genes in ingenuity networks	Function	Score[a]
1	*ABTB1*, *ARID3A*, *ARID3B, ATP6AP1L*, *B3GALT4*, *CBLN2*, Cebp, *CEBPG*, *DDX19B*, *DUS1L*, *FAM19A1*, *IFNGR*, *KLF13*, *MAN1A1*, *miR-125b-5p*, *OSBPL9*, *PCDHB10*, *PCTP*, peptidase, glucuronosyltransferase, *PERP*, *PPT2*, *ST18*, *TENM2*, *TNF*, *TOR2A*, *UGT*, *UGT2B15*, *UGT2B17*, *UGT2B28*, vacuolar H(+)-ATPase, GPD, 2′ 5′-oligoadenylate synthase, *VSIR*, and *ZNF385A*	cellular development, cell death and survival, cellular growth and proliferation	50
2	*CDKN1A*, *CDKN2A*, *CREB1*, *CSF1*, *CXCL8*, *DICER1*, *ERBB2*, *HIST*, *H4A*, *ID2*, *ITCH*, *KRAS*, *MAP2K7*, *miR-532-3p*, *NOTCH1*, *PTEFB*, *PAK5*, *PLK1*, *PTEN*, *RABL6*, *RB1,* RNA polymerase II, *SMARCA5*, trypsin, *UBE2I*, ubiquitin, ATPase, *E2F1*, and *IGF1R*	lipid metabolism, biochemistry of small molecules, development and function of the endocrine system	43
3	*ACSS1*, *ATAT1*, β catenin/TCF, *CAPN8*, *CNTN4*, *HDAC1/2*, *IKZF4*, *LATS*, *MDH2*, *MEF2D*, *TWIST*, *UVRAG*, *ZEB1*, *miR-135a-5p*, *TCF*, *miR-23a-3p*, PI3K class III, *miR-382-5p*, *POU4F2*, *SEPT3*, *TCF/LEF*, *miR-124*, *NLK*, *TP53*, and *TRPS1*	cellular development, developmental disorders, neurological disease	29

4	*ACVR1B*, α-1-antitrypsin, *CBFB*, *CDC2*, *CDK1*, Cyclin B, *GADD45*, *GCN5L*, *HIF1*, *ID1*, *KLHL20*, laminin family, *LIN28A*, *MIRLET7*, *RAD52*, *RPA*, *RUNX2*, *SMAD*, *SMAD2/3*, *SMAD4*, *SMAD5*, *TFIIH*, *TP63*, *DNA-PK*, *DUB*, *ESR1*, *PURA*, and *RSMAD*, *TGFBR2*, *TIMP3*, *BAP1*, and *MYLIP*	embryonic development, organ development, organismal development	24
5	*BAMBI*, *BMP*, *BNIP2*, calbindin, *CAMTA1*, *CASP8AP2*, caspase 3/6/7, *CIAP*, *COP9*, *CRIM1*, elastase, *ETS*, fascin, *IKB*, *IKK* (family), *IL1R*, *IRAK*, *IRF*, lymphotoxin, *MAP3K*, *miR-17-5p*, *miR-210-3p*, *NF-κB* complex, *NFκB-RelA*, *NFκB1-RelA*, *PKD2*, *RHEBL1*, *SDHD*, TNF receptor, *TP53I11*, *TRAF*, *TUSC2*, *UBE2*, *VSNL1*, and *ZBTB7A*	cancer, cell death and survival	20
6	*HSD*, *AKT*, *ATG2B*, *c-Src*, caveolin, *CYP19*, *ELMO2*, *FGF*, *FGFR*, *FGFRL1*, *FHOD1*, filamin, *FKHR*, *FOXF2*, Growth factor receptor, *HSPG*, integrin α4β1, integrin α5β1, *JINK1/2*, *LFA1*, *miR-130a-3p*, *miR-19b-3p*, *miR-200b-3p*, *PDGF* (family), *PPM1F,* presenilin, *PTK*, *RAB5*, *RERE*, *TSPAN8*, *WASF3*, *WDR37*, *WnT*, and *ZFPM2*	cell morphology, developmental disorders, skeletal and muscular disorders	20

[a]Scores > 3 were considered significant.

Table 2 Fold changes and activation in the top four upstream miRNAs in OSCC-derived cell lines

miRNA	Activation	Fold changes[a]			
		HSC2 /HNOKs	HSC3 /HNOKs	Ca922 /HNOKs	Ho-1-N1 /HNOKs
miR-125b-5p	inhibited	0.119	0.226	0.122	0.305
miR-17-5p	activated	3.067	1.718	2.139	3.235
miR-200b-3p	inhibited	0.117	0.209	0.227	0.554
miR-23a-3p	activated	4.161	5.157	1.703	1.710

[a]Fold changes for miRNA expression profiles data of OSCC-derived cell lines compared to HNOKs

Table 3 Gene ontology analysis of predicted gene targets of differentially expressed miRNAs in OSCC-derived cell lines

Molecular function
Top diseases and disorders
Cancer
Organismal injury and abnormalities
Tumor morphology
Inflammatory response
Developmental disorder
Top molecular and cellular functions
Cell death and survival
Cellular development
Cellular growth and proliferation
Cell cycle
Cellular movement

Figures

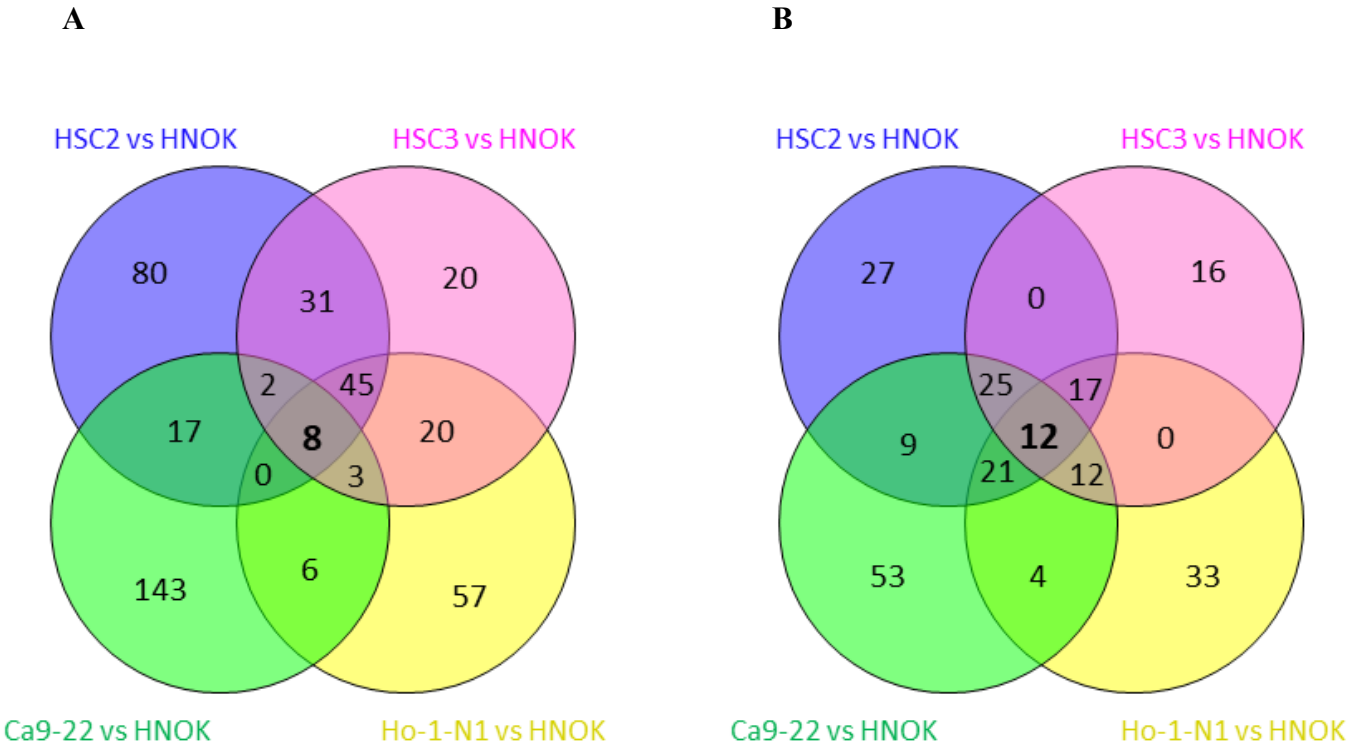

Fig. 1 Identification of differentially expressed exosomal miRNAs between OSCC-derived cell lines and HNOKs

A, Upregulated exosomal miRNAs; B, Downregulated exosomal miRNAs

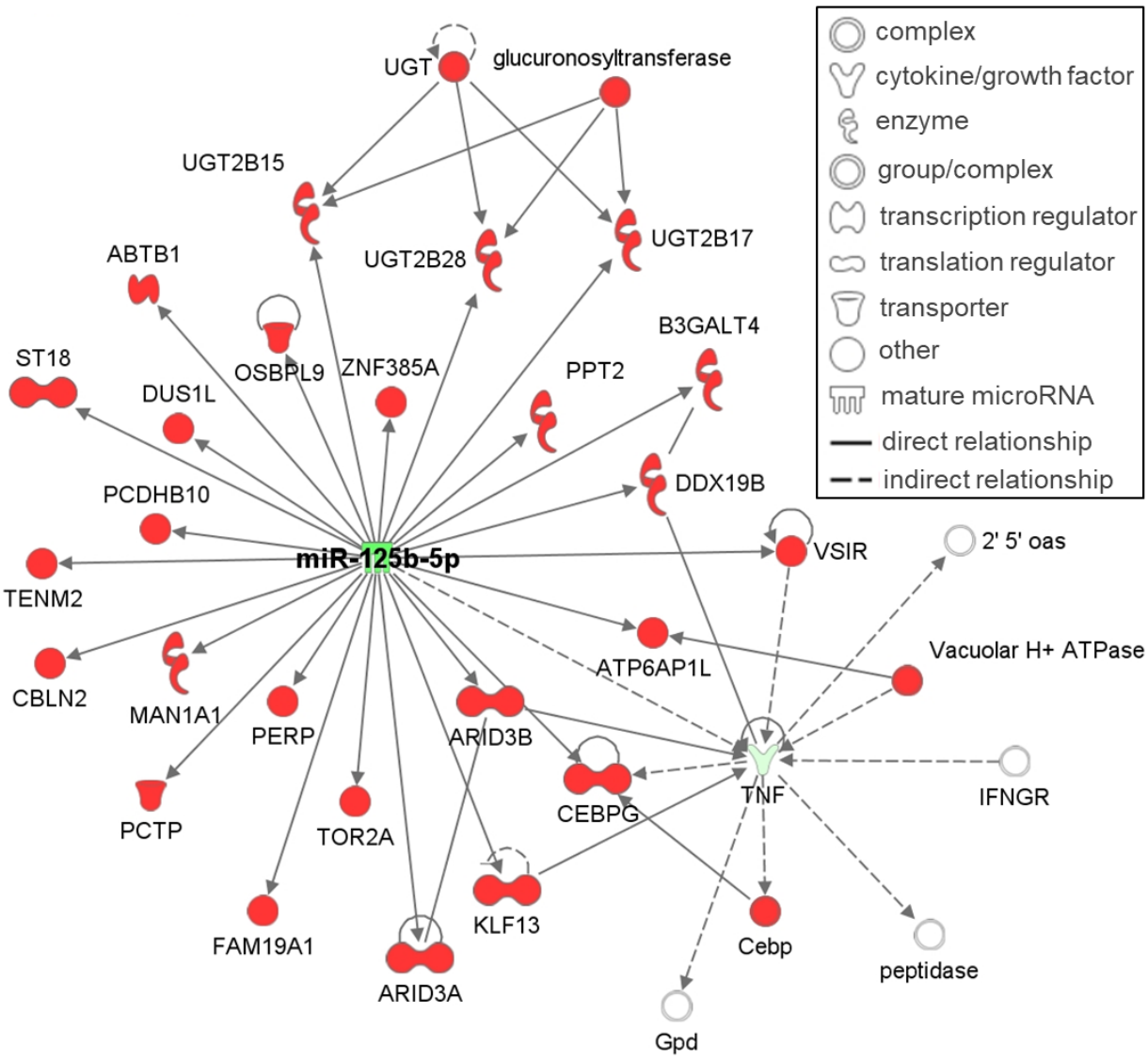

Fig. 2 The highest-scoring miRNA target network (network 1)

Functional relationships between dysregulated miRNAs and their target gene products based on known interactions are shown. Node shapes indicate the functional class of each gene product. The node color intensity indicates the degree of up- (red) and downregulation (green) in OSCC-derived cell lines compared with HNOKs.

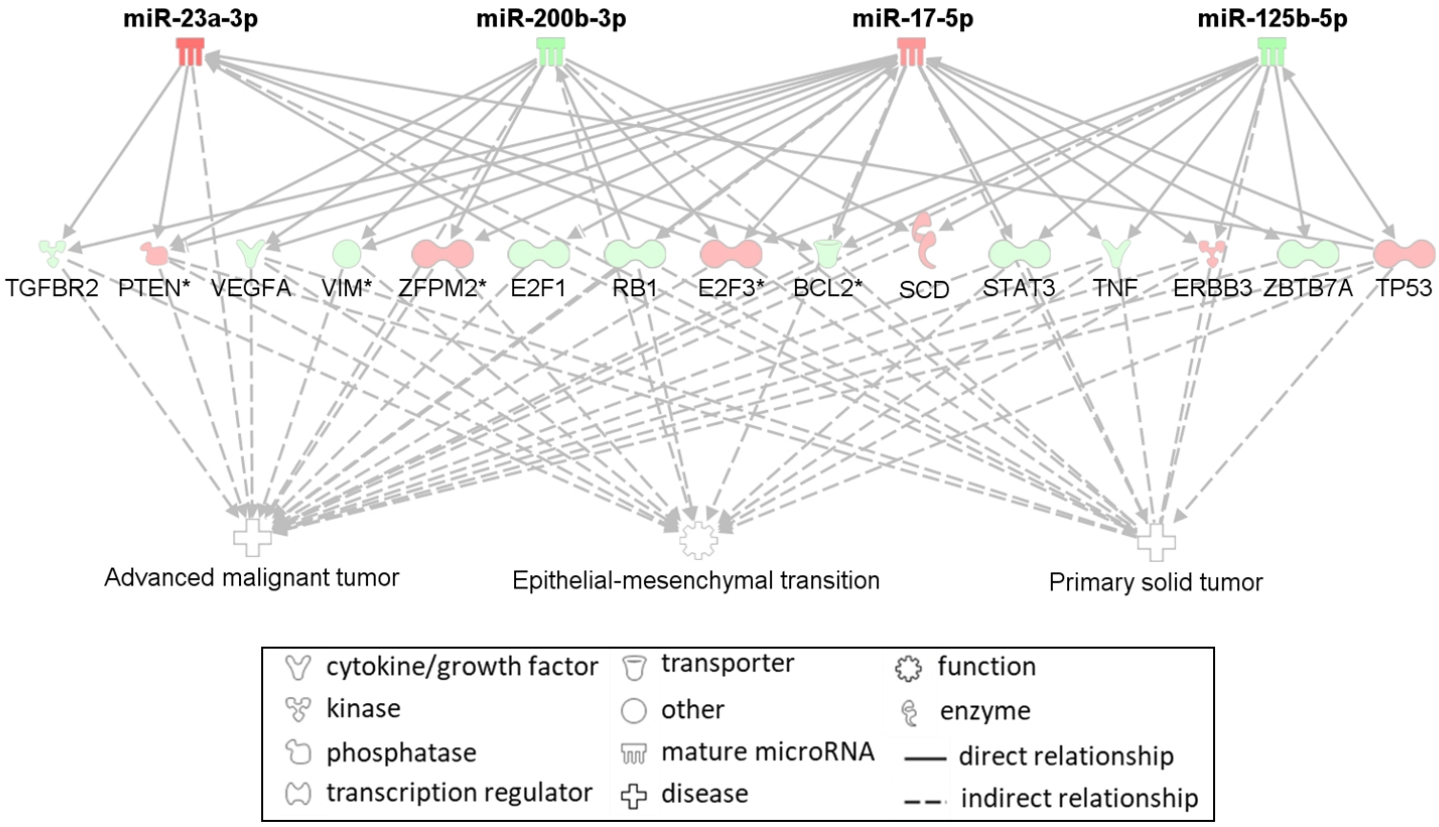

Fig. 3 Integrated network of the top four upstream miRNAs

A network containing miR-125b-5p, miR-17-5p, miR-200b-3p, and miR-23a-3p, their target genes, and their implicated biological processes was generated using IPA. Node shapes indicate the functional classes of the gene products. The node color intensity indicates the degree of up- (red) and downregulation (green) in OSCC-derived cell lines compared with HNOKs.

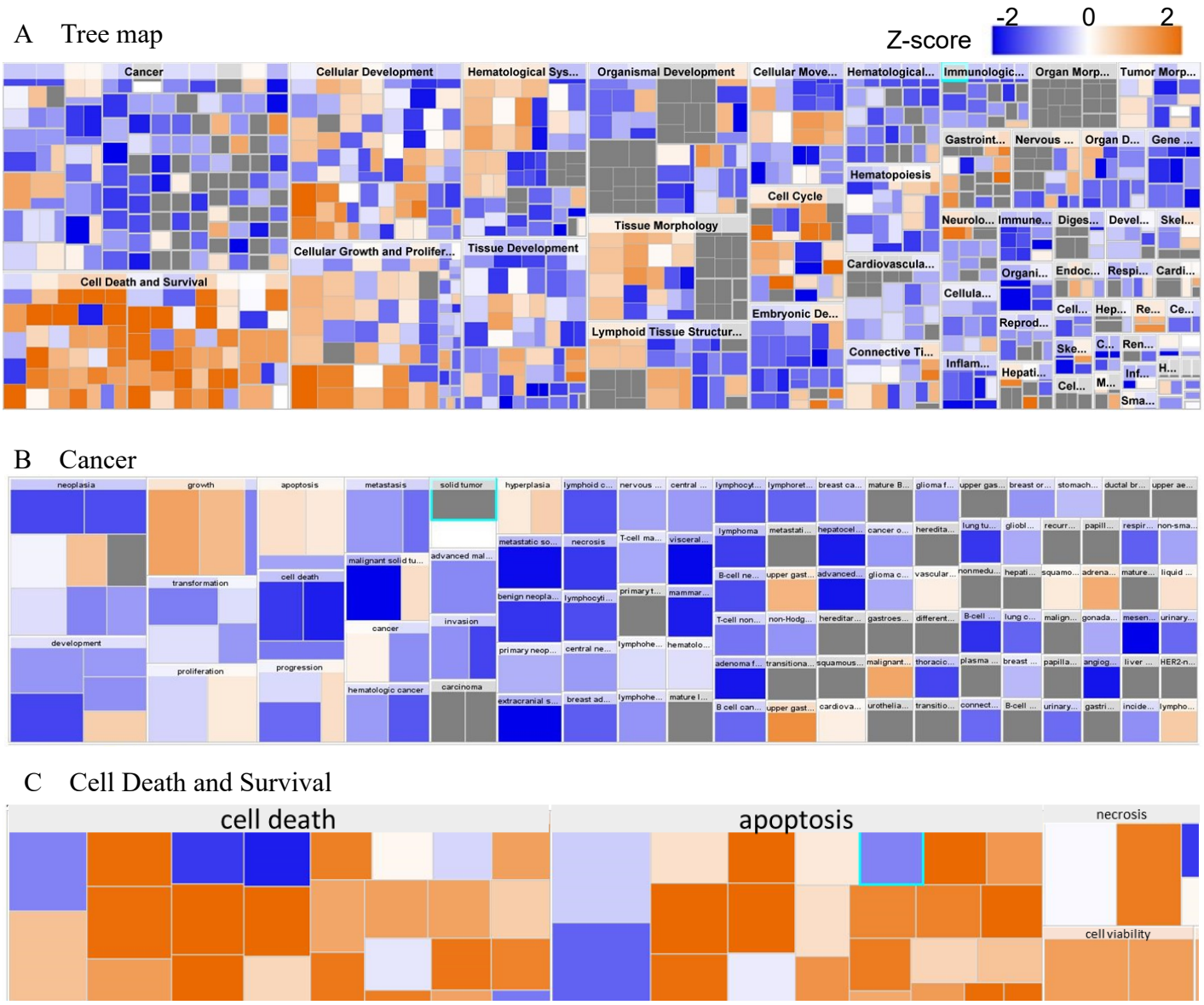

Fig. 4 Downstream effector analysis of differentially expressed exosomal miRNAs and their potential mRNA targets between OSCC-derived cell lines and HNOKs

A, Tree map (hierarchical heat map) depicting affected functional categories based on differentially expressed miRNAs and their identified potential genes where the major boxes represent a category of disease and functions; B, Cancer [Z-score = -2.06], C, Cell death [Z-score = 2.58] and apoptosis [Z-score = 2.86] in cell death and survival. Each individually colored rectangle is a particular biological function or disease, and the color range indicates its predicted activation state: increasing (orange), or decreasing (blue). Darker colors indicate higher absolute Z-scores. In this default view, the size of the rectangles is correlated with increasing overlap significance.

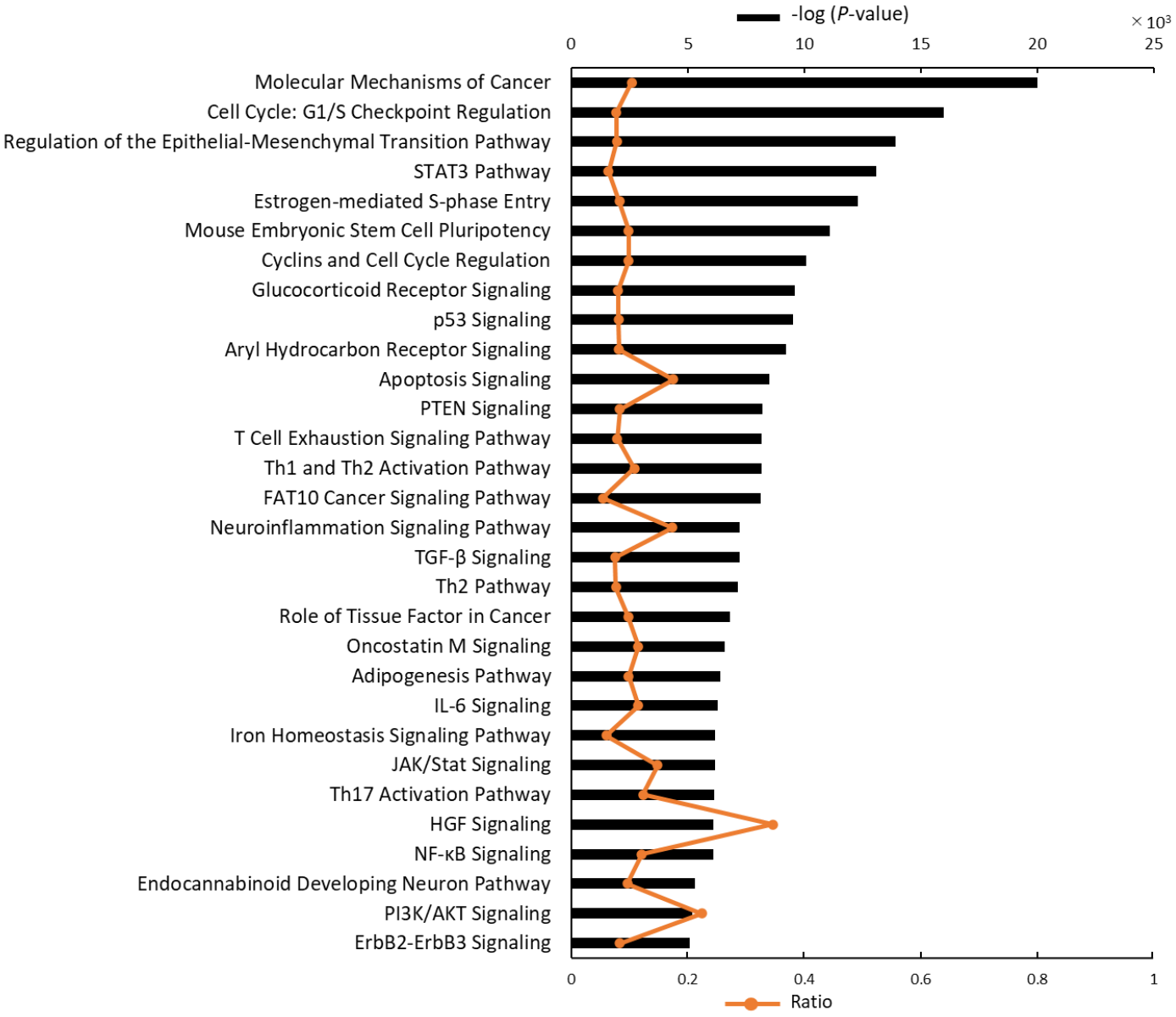

Fig. 5. Canonical pathways targeted by dysregulated miRNAs in OSCC-derived cell lines

The top 30 canonical pathways were evaluated by right-tailed Fisher's exact test to calculate the probability that the association is not explained by chance alone (black bars, upper y-axis). Enrichment ratios (i.e., the proportion of target genes in each pathway), referring to the population of selected genes from a pathway related to the total number of molecules that make up that particular pathway, are also shown (orange line graph, bottom y-axis).