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Down-regulation of the microRNA-99 family members in head and neck squamous cell carcinoma

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Abstract

Objectives—MicroRNA deregulation is a critical event in head and neck squamous cell carcinoma (HNSCC). Several microRNA profiling studies aimed at deciphering the microRNA signatures of HNSCC have been reported, but there tends to be poor agreement among studies. The objective of this study was to survey the published microRNA profiling studies on HNSCC, and to assess the commonly deregulated microRNAs in an independent sample set.

Materials and Methods—Meta-analysis of 13 published microRNA profiling studies was performed to define microRNA signatures in HNSCC. Selected microRNAs (including members of miR-99 family) were evaluated in an independent set of HNSCC cases. The potential contributions of miR-99 family to the tumorigenesis of HNSCC were assessed by *in vitro* assays.

Results—We identified 67 commonly deregulated microRNAs. The up-regulation of miR-21, miR-155, miR-130b, miR-223 and miR-31, and the down-regulation of miR-100, miR-99a and miR-375 were further validated in an independent set of HNSCC cases with quantitative RT-PCR. Among these validated microRNAs, miR-100 and miR-99a belong to the miR-99 family. Our *in vitro* study demonstrated that restoration of miR-100 to the HNSCC cell lines suppressed cell proliferation and migration, and enhanced apoptosis. Furthermore, ectopic transfection of miR-99

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family members down-regulated the expression of insulin-like growth factor 1 receptor (IGF1R) and mechanistic target of rapamycin (mTOR) genes.

Conclusion—In summary, we described a panel of frequently deregulated microRNAs in HNSCC, including members of miR-99 family. The deregulation of miR-99 family contributes to the tumorigenesis of HNSCC, in part by targeting IGF1R and mTOR signaling pathways.

Keywords

meta-analysis; HNSCC; microRNA profiling; miR-99 family; miR-100; IGF1R; mTOR; tumor suppressor

Introduction

Head and neck cancer, predominantly head and neck squamous cell carcinoma (HNSCC), is the sixth most common cancer in the world, with an incidence of approximately 600,000 cases per year and a mortality rate of approximately 50%¹. Despite the improvements in surgery, radiotherapy and chemotherapy, the prognosis for HNSCC patients has not significantly improved for the past 3 decades. Improvement in patient survival rate requires better understanding of the initiation and progression of HNSCC, so that aggressive tumors can be detected early in the disease process and targeted with appropriate therapeutic interventions. While attempts have been made to identify genomic alterations that contribute to the tumorigenesis of HNSCC, most efforts are focused on protein coding genes. Current knowledge of genomic aberrations associated with non-coding genes (e.g., microRNA) and their contributions to the onset and propagation of HNSCC is relatively limited.

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs that control the target gene's expression at the post-transcriptional level. Several microRNAs have been functionally classified as proto-oncogenes or tumor suppressors. Dysregulation (e.g., overexpression or loss of expression) of these "cancerous" microRNAs contributes to tumor initiation and progression by promoting uncontrolled proliferation, favoring survival, and/or promoting invasive behavior^{2,3}. MicroRNA deregulation is a frequent event in HNSCC. A number of microRNA profiling studies aimed at deciphering the microRNA signatures of HNSCC have been reported, but there tends to be poor agreement among them. This study seeks to identify and validate the microRNA candidates associated with HNSCC. We carried out a comprehensive meta-analysis on 13 published microRNA profiling studies on HNSCC, and then assessed the most frequently observed microRNA alterations (including members of miR-99 family) in an independent sample set. The contributions of miR-99 family members in HNSCC were further confirmed using in vitro model.

Materials and Methods

Literature search

To identify relevant literature, a PubMed search was performed for microRNA profiling studies that used human tissue samples obtained from surgically resected HNSCC and corresponding non-cancerous tissues from the oral cavity and laryngopharynx. Studies were included in the systematic review if: 1) they were microRNA profiling studies in patients with HNSCC; 2) they used HNSCC and adjacent non-cancerous tissues for comparison; 3) they used large-scale microRNA profiling techniques (e.g., microarrays or TaqMan qRT-PCR arrays); 4) they were published as full articles in English. Studies using HNSCC cell lines, serum or saliva samples, or focused on specific disease stages, or using other microRNA techniques were not included. Review articles were not included.

Differentially expressed microRNAs were searched from these microRNA profiling studies. MicroRNAs were then ranked as described by Griffith et. al.,⁴ and Chan et. al.,⁵ based on the following criteria: 1) the microRNA was consistently reported as differentially expressed; 2) the reported differential expression was in a consistent direction of change; 3) the frequency of the differential expression of a microRNA reported in the studies surveyed.

Tumor procurement and RNA extraction

Surgically resected archived frozen tissue samples from 10 cases of HNSCC from the oral tongue were obtained from the Cooperative Human Tissue Network (CHTN), Midwestern Division (Ohio State University). Clinical characterization of the HNSCC patients is summarized in Supplementary Table 1. This study was approved by Institutional Review Board at the University of Illinois at Chicago. Tissue samples were obtained after the tumor resection and snap frozen. Specimens containing more than 80% tumor cells based on H&E pathological examination were selected for microdissection under a microscope by a pathologist (X.L.). The total RNA was isolated using miRNeasy Mini kit (Qiagen), and quantified by the RiboGreen RNA Quantitation Reagent (Molecular Probes).

Quantitative RT-PCR analysis

The relative expression levels of miR-21, miR-155, miR-130b, miR-223, miR-31, miR-7, miR-34b, miR-100, miR-99a, miR-99b, miR-375, and miR-125b were determined using TaqMan microRNA assays per the manufacturer's protocol (Applied Biosystems). The relative mRNA levels of IGF1R and mTOR were examined using a quantitative two-step RT-PCR assay with gene specific primer sets (OriGene) as described previously⁶. Quantitative PCR reactions were performed using a 7900HT Fast Real-Time PCR system (Applied Biosystems). The relative microRNA and mRNA levels were computed using the $2^{-\Delta\Delta C_t}$ analysis method⁷, where U6 or actin was used, respectively, as an internal reference.

Cell Culture and the treatments of cell lines

The 1386Ln cell line was derived from a patient with HNSCC of hypopharynx⁴⁴. The UM1 cell line was derived from a patient with HNSCC of the tongue⁴⁵. These cell lines were gifts from Dr. P.G. Sacks of the New York University and Dr. D.T. Wong of the University of California at Los Angeles. These cell lines were maintained in DMEM/F12 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (GIBCO). For functional analysis, miR-99a, miR-99b, miR-100 mimics and non-targeting microRNA mimics (Dharmacon) were transfected into cells using DharmaFECT Transfection Reagent 1 as previously described⁸. The transfection was confirmed by quantitative RT-PCR analysis as described above, and an over 100-fold increase of transfected microRNA was observed.

Proliferation, apoptosis and cell migration assays

Cell proliferation was measured by MTT assay as previously described⁹, with minor modification. In brief, 48 h post transfection, medium in each well was replaced by 100 µl of fresh serum-free medium with 0.5 g/L MTT. After incubation at 37 °C for 4 h, the MTT medium was aspirated out, and 50 µl of DMSO was added to each well. After incubation at 37 °C for additional 10 min, the absorbance value of each well was measured using a plate reader at a wavelength of 540 nm. The apoptosis was measured using the Annexin V-FITC Apoptosis Detection Kit (Invitrogen) and measured with a flow cytometer (FACScalibur, Becton–Dickinson) as previously described¹⁰. Cell migration was measured using a wound healing assay as described previously¹¹. In brief, cells were seeded in 12-well plates and cultured to confluence. Wounds of 1 mm width were created with a plastic scribe, and cells were washed and incubated in a serum-free medium. 24 hours after wounding, cultures were

fixed and observed under a microscope. A minimum of 5 randomly chosen areas were measured.

Western blot analysis

Western blots were performed as described previously¹² using antibodies specific to mTOR, IGF1R (Cell Signaling), beta-actin (Sigma-Aldrich), and a Immuno-Star HRP Substrate Kit (BIO-RAD, USA).

Results and Discussion

In previous studies, the microRNA profiles of HNSCC have been investigated using microarrays and quantitative RT-PCR based approaches. A common drawback of these studies is the lack of agreement among them. A number of potential factors may contribute to the observed inconsistency, such as the heterogeneity in the tissue samples, variations in genetic and environmental backgrounds of the subjects, and the differences in profiling technologies. Although reanalysis of the profiling data as a whole remains a challenge, meta-analysis of multiple studies is a reasonable approach for identifying consistently-reported, differentially-expressed microRNAs in HNSCC. Based on the inclusion and exclusion criteria, 13 independent microRNA profiling studies on HNSCC (Table 1) were selected for the meta-analysis. A total of 432 differentially expressed microRNAs were reported in these studies, including 264 up-regulated and 168 down-regulated microRNAs. Among the reported differentially expressed microRNAs, 90 were reported by at least 2 studies, 67 (74.4%) with a consistent direction of change (Supplementary Table 2 and 3), and 23 (25.6%) with an inconsistent direction (Supplementary Table 4). Among the 67 microRNAs with consistent directions, 46 (68.7%) were reported to be up-regulated (Supplementary Table 2), and 21 (31.3%) were reported to be down-regulated (Supplementary Table 3) in HNSCC. As shown in Table 2, 11 differentially expressed microRNAs were reported in at least 4 studies with consistent direction, including 7 consistently up-regulated microRNAs (miR-21, miR-155, miR-130b, miR-31, miR-223, miR-34b, miR-7), and 4 consistently down-regulated microRNAs (miR-100, miR-99a, miR-125b, miR-375) in HNSCC.

To further evaluate the deregulation of these 11 microRNAs in HNSCC, the levels of these microRNAs were determined in a panel of HNSCC (n = 10) along with their matching normal control samples by quantitative RT-PCR. As shown in Figure 1, the levels of miR-21, miR-155, miR-130b, miR-31 and miR-223 in HNSCC were significantly higher than those in normal control samples ($p < 0.05$). The levels of miR-100, miR-99a and miR-375 were statistically significantly lower than those in normal control samples ($p < 0.05$). An apparent down-regulation of miR-125b was also observed; however the difference was not statistically significant ($p = 0.06$). These observations are in agreement with the previous studies. Among these validated microRNAs, miR-100 and miR-99a belong to the miR-99 family. It is worth noting that miR-99b (the 3rd member of the miR-99 family) is also down-regulated in our HNSCC cases ($p < 0.05$). Nevertheless, our sample size is relatively small, and additional validation studies with larger sample size will be needed to confirm these observations.

Based on bioinformatics prediction, these deregulated microRNAs (miR-21, miR-155, miR-130b, miR-31, miR-223, miR-100, miR-99a and miR-375) target a number of common molecular pathways that contribute to tumorigenesis, including the Wnt signaling pathway, mTOR signaling pathway, TGF-beta signaling pathway, and MAPK signaling pathway (Supplementary Table 5). In fact, several of these microRNAs have been functionally linked with the initiation and progression of the HNSCC or other forms of solid tumors. The up-regulation of miR-21 is the most consistently observed microRNA deregulation (reported in

11 out of 13 studies we surveyed). It has been linked with poor prognosis in HNSCC and may act as an apoptosis inhibitor^{13,14}. MiR-155 acts as an oncogene in breast cancer and regulates cell survival, growth, and chemosensitivity^{15–17}. MiR-130b has been shown to promote liver tumor-initiating cell growth and self-renewal¹⁸, and targets tumor suppressor RUNX3 in gastric cancer¹⁹. The role of miR-31 appears to be cancer type specific; while miR-31 inhibits metastasis in breast cancer²⁰, up-regulation of miR-31 is essential to the TGF-beta-induced invasion and metastasis of colon cancer cells²¹. As shown in our meta-analysis, up-regulation of miR-31 is frequently observed in HNSCC. Increases in plasma miR-31 have recently been suggested as a potential marker of oral cancer²². MiR-223 promotes gastric cancer invasion and metastasis²³, and elevated serum miR-223 may serve as a biomarker for hepatocellular carcinoma²⁴. Frequent down-regulation of miR-375 has also been reported in gastric cancer, and has been shown to inhibit cell proliferation and regulate cell survival^{25,26}.

Deregulation of miR-99 family (miR-99a/b and miR-100) has also been reported in several cancer types^{27,28}. However, their role(s) in HNSCC are not well defined. UM1 and 1386Ln are HNSCC cell lines that exhibit reduced levels of miR-100 and miR-99a (Supplementary Figure 1). As shown in Figure 2A, ectopic transfection of miR-100 mimic to UM1 and 1386Ln cells led to statistically significant down-regulation of cell proliferation as compared to the cells treated with control mimic. A statistically significant increase in apoptosis was observed when cells were treated with miR-100 mimic (Figure 2B). Similar observations of proliferation and apoptosis were made when cells were transfected with miR-99a or miR-99b (data not shown). Ectopic transfection of miR-100 mimic to UM1 also led to reduced cell migration (Figure 2C). An apparent reduction of cell migration was also observed in 1386Ln cells that were treated with miR-100 mimic. However the difference was not statistically significant. Our results, together with earlier observations in other cancer types^{27,28}, suggest that miR-99 family members are important tumor suppressors that regulate proliferation, apoptosis and migration.

A number of recent functional studies suggested that the members of miR-99 family target insulin-like growth factor 1 receptor (IGF1R) and mechanistic target of rapamycin (mTOR) signaling pathways^{29–31}. As shown in Figure 3A, decreases in IGF1R protein level were observed in both UM1 and 1386Ln cells that were treated with miR-100, miR-99a or miR-99b. Decreases in mTOR protein level were observed in UM1 cells treated with miR-100 or miR-99a, and in 1386Ln cells treated with miR-100, miR-99a or miR-99b. No apparent change in mTOR level was detected in UM1 cells treated with miR-99b. Significant reductions in IGF1R and mTOR mRNA levels were observed in both UM1 and 1386Ln cells that were treated with miR-100, miR-99a or miR-99b as measured by quantitative RT-PCR (Figure 3B). These results are in agreement with previous findings showing that the deregulations of miR-99 family members contribute to the tumorigenesis, in part by targeting IGF1R and mTOR^{29–31}. The targeting sequences for miR-100, miR-99a and miR-99b have been identified in the 3'-UTR of the mRNAs for IGF1R and mTOR genes (Supplementary Figure 2). Direct interactions among the miR-99 members and these targeting sequences in IGF1R and mTOR have been functionally confirmed by multiple studies using reporter gene assays^{31,46–48}. It is worth noting that overexpression of IGF1R and activation of mTOR signaling have been observed in HNSCC, and are often associated with poor prognosis^{49–52}. A number of targeted therapies have been under investigation for several types of solid tumors^{53,54}. Thus, our results, together with previous observations, provided rational for developing potential microRNA-based therapy for HNSCC.

In summary, we described a panel of frequently deregulated microRNAs in HNSCC, including the up-regulation of miR-21, miR-155, miR-130b, miR-31 and miR-223, and the down-regulation of miR-100, miR-99a/b and miR-375. The deregulation of miR-99 family

(miR-99a/b and miR-100) contributes to the tumorigenesis of HNSCC, in part by targeting IGF1R and mTOR signaling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HNSCC	head and neck squamous cell carcinoma
IGF1R	insulin-like growth factor 1 receptor
miRNA	MicroRNA
mTOR	mechanistic target of rapamycin
RT-PCR	Reverse transcription polymerase chain reaction

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Figure 1. Deregulation of microRNAs in HNSCC

The qRT-PCR was performed to assess the levels of miR-21 (A), miR-155 (B), miR-130b (C), miR-223 (D), miR-31 (E), miR-7 (F), miR-34b (G), miR-100 (H), miR-99a (I), miR-99b (J), miR-375 (K), and miR-125b (L) in paired HNSCC tissue samples and normal control tissue samples (n = 10). The p-values were computed using one-way ANOVA. The boxes represent 25th to 75th percentile of the observations, and the lines in the middle of the box represent the median. The whiskers represent maximum (or minimum) observations below (or above) the 1.5 times of the interquartile range, respectively. Outliers are also indicated in the plots as circles.

Figure 2. The effect of miR-100 on proliferation, apoptosis and migration in HNSCC cells
 The miR-100 mimic and negative control microRNA were introduced into the UM1 and the 1386Ln cells. Cell proliferation (A), apoptosis (B) and migration (C) were measured as described in the Material and Methods section. Data represents at least 3 independent triplicate experiments with similar results. * indicates $p < 0.05$.

A **B**

Figure 3. The effect of miR-99 family on the expression of IGF1R and mTOR in HNSCC cells
 The UM1 and 1386Ln cells were treated with miR-99a, miR-99b, miR-100 or negative control mimic. The levels of IGF1R and mTOR proteins were examined by Western blot analyses (A). The relative levels of IGF1R and mTOR mRNAs were examined by qRT-PCR (B). Data represents at least 3 independent experiments with similar results. * indicates $p < 0.05$.

Table 1Thirteen microRNA profiling studies used in the meta-analysis ^a

Reference	Sample size		site	Profiling platform
	HNSCC	Normal		
Wong TS, 2008 Clin Cancer Res, ³²	4	4	tongue	TaqMan MicroRNA assay (Applied Biosystems)
Chang SS, 2008 Int J Cancer, ³³	4	4	2 base of tongue, 1 layngeal, 1 tonsillar	microRNA array (Genesensor)
Li J, 2009 Clin Cancer Res, ^{14 b}	10	10	tongue	microRNA array (probe set from Invitrogen)
Avissar M, 2009 Clin Cancer Res, ³⁴	16	5	not specified	microRNA array (Ambion)
Ramdas L, 2009 Head Neck, ³⁵	5	5	2 tongue, 1 base of tongue, 1 laryngopharynx, 1 FOM	microRNA array (Ambion)
Childs G, 2009 Am J Pathol, ³⁶	8	8	not specified	microRNA array (probes from Ambion)
Cervigne NK, 2009 Hum Mol Genet, ³⁷	12	7	8 tongue, 2 lateral tongue, 1 buccal mucosa, 1 FOM	TaqMan MicroRNA assay (Applied Biosystems)
Liu CJ, 2010 Cancer Res, ³⁸	10	10	not specified	TaqMan MicroRNA assay (Applied Biosystems)
Hui AB, 2010 Clin Cancer Res, ³⁹	54	4	20 Larynx, 24 oropharynx, 10 hypopharynx	TaqMan MicroRNA assay (Applied Biosystems)
Kikkawa N, 2010 Br J Cancer, ⁴⁰	10	10	hypopharyngeal	TaqMan MicroRNA assay (Applied Biosystems)
Scapoli L, 2010 Int J Immunopathol Pharmacol, ⁴¹	15	11	8 jaw, 5 tongue, 1 cheek, 1 FOM	microRNA array (Invitrogen)
Lajer CB, 2011 Br J Cancer, ^{42 c}	49	39	30 oral cavity, 19 oro- or hypopharynx	microRNA array (Affymetrix)
Rentoft M, 2011 Int J Oncol, ⁴³	21	8	tongue	microRNA array (Exiqon)

^a: Our meta-analysis included studies on SCC from oral cavity and laryngopharynx. HNSCC from other anatomical sites (e.g., nasal cavity, external ear) were not included.

^b: Differentially expressed microRNAs identified from both early-stage TSCC and advanced-stage TSCC patients from Li J, 2009 Clin Cancer Res study were included in the analysis.

^c: Differentially expressed microRNAs identified from both OSCC and PSCC patients from Lajer CB, 2011 Br J Cancer study were included in the analysis.

Table 2

Differentially expressed microRNAs that were consistently reported in HNSCC (by at least 4 studies)

MicroRNA	Chromosomal location	Mature miR sequence	Up- / down-regulation	References	No. of refs.	Sample size (SCC/ctrl)
miR-21	17q23.1	uagcuuaucaagacugauguuga	Up	14,32–34,36–42	11	192 / 112
miR-155	21q21.3	uuauugcuaauucgugauagggu	Up	32,33,37–39,42	6	133 / 68
miR-130b	22	cagugcaaugaugaaaggccau	Up	34,38,39,42	4	129 / 58
miR-223	Xq12	ugucaguuugucaaaauaccca	Up	37–39,42	4	125 / 60
miR-31	9p21.3 9q21.32 or	aggcaagaugcuggcuaugcu	Up	14,32,38,42	4	73 / 63
miR-7	15q26.1 or 19p13.3	uggaagacuagugauuuuuguuu	Up	35,37,40,43	4	48 / 30
miR-34b	11q23.1	caaucuauacuccacugccau	Up	32,35,37,38	4	31 / 26
miR-100	11q24.1	aaccgguagauccgaaucuuug	Down	32,38–40,42	5	127 / 67
miR-99a	21q21.1	aaccgguagauccgaaucuuug	Down	32,38–40,42	5	127 / 67
miR-375	2q35	uuuguuucguucggcucgcguga	Down	34,39,40,42	4	129 / 58
miR-125b	11q24.1 or 21q21.1	uccuugagaccuuaucuuuga	Down	32,39,40,42	4	117 / 57