

Distinctive pattern of let-7 family microRNAs in aggressive carcinoma of the oral tongue in young patients

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Abstract. Oral cavity squamous cell carcinoma may be more aggressive at presentation and recurrence in young patients compared with older patients. Dysregulation of microRNAs (miRNAs or miRs) has been associated with the development and prognosis of oral cavity cancer. The present study investigated miRNA expression in carcinoma of the oral tongue in young patients. miRNA expression profiles were evaluated in formalin-fixed, paraffin-embedded samples of tumor and normal mucosa from 12 patients aged <30 years old with squamous cell carcinoma of the tongue. The levels of let-7f-5p, miR-30b-5p and let-7e-5p were upregulated in tumors (P<0.05). The expression of let-7f-5p was upregulated in non-aggressive tumors, while the expression of let-7e-5p was upregulated in aggressive tumors, compared with the corresponding normal tissue. Aggressive tumors had higher levels of let-7c, miR-130a-3p, miR-361-5p, miR-99a-5p, miR-29c-3p and let-7d-5p than non-aggressive tumors (P<0.05). The findings remained significant for let-7c upon false-discovery rate correction. An excellent correlation was noticed on validation of NanoString counts by quantitative polymerase chain reaction. The comparison with published findings in adults demonstrated a unique miRNA signature in young patients with aggressive disease. Aggressive oral cavity cancer in patients <30 years old is associated with a distinctive expression pattern of the let-7 family. Larger studies including direct comparison with older patients are warranted.

Introduction

Squamous cell carcinoma (SCC) of the oral tongue is one of the most common types of head and neck cancer (1,2). The incidence peaks in the sixth decade of life (1), and is clearly associated with ethylism and smoking (3). Previous studies suggest that, although the overall incidence of head and neck cancers is decreasing in the USA, the incidence of oral tongue carcinoma specifically is increasing in young patients (2,4-7), particularly in white women (8). The factors responsible for this shift remain unclear. Unlike oropharyngeal cancer (malignancy of the tonsils or the base of tongue), whose rise in young patients has been attributed to malignant transformation subsequent to human papilloma virus infection (9,10), no such association has been observed for oral tongue SCC.

The prognostic significance of age at diagnosis of oral tongue SCC is controversial. Garavello *et al* (11) demonstrated that patients younger than 40 years are at higher risk of recurrence and mortality than matched older patients. Other studies revealed that the prognostic disadvantage of young age may be limited to individuals with advanced, as opposed to early, disease at presentation (12) or with recurrent, as opposed to new-onset, disease (13). Accordingly, we previously reported that tongue carcinoma in patients <30 years old is more advanced at presentation, with significantly high rates of regional metastases and an increased risk of distant failure compared with patients >30 years (14). In addition, recurrent disease is aggressive, with a fatality rate of 100% (14). However, other authors noticed that clinical outcomes in young patients were similar to (15-18) or even better than (19-21) those in older patients.

MicroRNAs (miRNAs or miRs) are short, non-coding RNA molecules that regulate gene expression by controlling messenger (m)RNA translation (22). They are considered to play a role in normal biological processes, from cell growth to cell differentiation and death (22). miRNAs are dysregulated in various malignancies, and can act as both oncogenes and tumor suppressors (22,23). In the head and neck, specific patterns of miRNA expression have been detected in cell lines and tumoral tissues from the hypopharynx, oral cavity, larynx, nasopharynx and salivary glands (23). Specific miRNAs that have been implicated in oral SCC cell lines and tumoral tissues include miR-211 (22), miR-184 (24), miR-137, miR-193a (25),

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miR-133a, miR-133b (26), miR-98 (27), *Homo sapiens* (hsa)-miR-15a (28), miR-126 (29) and miR-155 (30). Certain patterns of miRNA expression can also serve as prognostic markers. For example, high expression of miR-211 (22) and miR-21 (31,32) was associated with poor prognosis in patients with oral carcinoma.

The aim of the present study was to identify specific miRNAs that are dysregulated in SCC of the oral tongue in young patients and to determine if these aberrations resemble known miRNA profiles in older patients.

Materials and methods

Study design and patient characteristics. The present study was approved by the Institutional Review Board of Rabin Medical Center (Petah Tikva, Israel), with waiver of informed consent.

The study group included 12 patients aged ≤ 30 years with SCC of the oral tongue who underwent partial glossectomy with neck dissection at Rabin Medical Center between January 1996 and December 2013. The mean age of the patients was 22.5 years (range, 15-30 years), and 7 (58%) patients were females. Two patients were smokers (1 and 6 packs/year, respectively), and none consumed alcohol. The mean follow-up time was 56 months.

At the time of diagnosis, 10 patients had advanced disease (stage 3-4), 9 had regional lymph node involvement and none had systemic metastases. All patients with advanced disease were also treated with radiotherapy, and 8 received adjuvant chemotherapy.

Five patients had a recurrence of the disease. The mean time from diagnosis to recurrence was 11.4 months (range, 5-16 months). All patients succumbed to the disease within a mean time of 18.4 months (range, 14-28 months). These patients were categorized as having aggressive disease, and the other 7 patients, without recurrence, were categorized as having non-aggressive disease. The mean follow-up time of the group with non-aggressive disease was 83 months (range, 24-132 months).

Sample collection. All tumors were diagnosed following partial glossectomy. For the present study, all available hematoxylin and eosin-stained slides were reviewed by a head and neck pathologist, and paraffin-embedded blocks of the most representative slides were used for RNA extraction. Samples of tumoral tissue and peritumoral normal tongue tissue were collected for each patient.

Total RNA was extracted from formalin-fixed, paraffin-embedded samples using RecoverAll Total Nucleic Acid Isolation kit, according to the manufacturer's protocol (Ambion, Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and further purified with phenol-chloroform, followed by ethanol precipitation. The final RNA concentration and purity were measured with an ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA).

miRNA profiling. In total, >800 human miRNAs were profiled with the multiplexed nCounter[®] miRNA Expression Assay kit (NanoString Technologies, Seattle, WA, USA), according to

the manufacturer's protocol. Briefly, 100 ng of total RNA was used as input material with 3 μ l of 3-fold-diluted sample. A specific DNA tag was ligated onto the 3' end of each mature miRNA for exclusive identification for each of the miRNA species present in the sample. Tagging was performed in a multiplexed ligation reaction utilizing reverse complementary bridge oligonucleotides (NanoString Technologies). All hybridization reaction mixtures were incubated at 64°C for 18 h. Excess tags were then removed, and the resulting material was hybridized with a panel of fluorescently labeled, bar-coded reporter probes, which were specific to the miRNA of interest. miRNAs abundance was quantified with the nCounter[®] Prep Station (NanoString Technologies) by counting the individual fluorescent barcodes and identifying the target miRNA molecules present in each sample. Each sample was normalized to the geometric mean of the top 100 highest expressed miRNAs. The mean value of the negative controls was set as the lower threshold for each sample; thus, when $\geq 50\%$ of the value was equal to or lower than the lower threshold, the miRNA was excluded. Using these criteria, 93 miRNAs were identified to be eligible for subsequent analyses.

Quantitative polymerase chain reaction (qPCR). qPCR was performed to validate the top candidates obtained with the nCounter[®] miRNA Expression Assay kit, according to the manufacturer's protocol (NanoString Technologies). The TaqMan Universal PCR Master Mix, Nno AmpErase UNG (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to test miRNA expression. PCR amplification and reading were performed with the StepOne[™] Real-Time PCR System (Life Technologies; Thermo Fisher Scientific, Inc.) under the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. Expression values were calculated using the comparative threshold cycle method (33), with normalization according to the total cellular RNA content.

Statistical analysis. Following data pre-processing and normalization, differential expression analysis was performed using nSolver[™] Analysis Software (version 2.0; NanoString Technologies) and EXpression Analyzer and DisplayER version 6.06 ([//acgt.cs.tau.ac.il/expander/](http://acgt.cs.tau.ac.il/expander/)). Student's *t*-test was used for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference. *P*-values were adjusted according to the false-discovery rate (FDR).

Results

Comparisons. A total of 24 samples were analyzed, one from tumor and one from adjacent healthy tissue, for each patient. The miRNA results were compared between tumor and normal tissues, and between aggressive and non-aggressive tumors. No significant differences were identified in demographic characteristics or tumor stage between patients with aggressive and non-aggressive tumors ($P = 0.87$ and $P = 0.47$, respectively). Table I contains the significant differences in miRNA expression between the groups.

Tumors vs. adjacent normal tissues. Comparisons of the 12 tumor samples (aggressive and non-aggressive combined)

Table I. miRNA expression by the studied groups.

Comparison	miRNA	Fold-change	P-value
Tumors vs. healthy tissue			
Upregulation	let-7f-5p	1.54	0.015 ^a
	miR-30b-5p	1.49	0.039
	let-7e-5p	1.27	0.040
	miR-26a-5p	1.51	0.086
Downregulation	miR-185-5p	0.78	0.060
Aggressive tumors vs. non-aggressive tumors			
Upregulation	let-7c	1.85	0.005 ^a
	miR-130a-3p	1.39	0.008
	miR-361-5p	1.47	0.021
	let-7d-5p	1.45	0.022
	miR-99a-5p	1.67	0.025
	miR-29c-3p	2.29	0.043
	let-7f-5p	1.49	0.051
Downregulation	miR-181a-5p	0.46	0.069
	let-7i-5p	0.67	0.075
Normal tissue adjacent to aggressive vs. non-aggressive tumors			
Upregulation	miR-107	1.86	0.010 ^a
	let-7f-5p	1.72	0.016
	miR-30b-5p	1.40	0.062
Downregulation	miR-34a-5p	0.67	0.015

^aFalse-discovery rate-adjusted $P < 0.05$. miR/miRNA, microRNA.

with the 12 normal-tissue samples revealed an upregulation of let-7f-5p, miR-30b-5p and let-7e-5p in the tumors ($P < 0.05$); the difference for let-7f-5p remained significant following FDR correction. Stratification by disease aggressiveness yielded an upregulation of let-7f-5p in non-aggressive tumors and of let-7e-5p in aggressive tumors, compared with the corresponding healthy-tissue samples.

Aggressive vs. non-aggressive tumors. Fig. 1 represents the differential expression of the miRNAs with the highest expression levels, while Fig. 2 represents the expression of the significantly altered miRNAs in aggressive vs. non-aggressive tumors. There was a significant upregulation of let-7c, miR-130a-3p, miR-361-5p, miR-99a-5p, miR-29c-3p and let-7d-5p in the aggressive tumors ($P < 0.05$); the difference for let-7c remained significant after FDR correction. Additionally, the aggressive tumors exhibited downregulation of miR-181a-5p and let-7i-5p ($P = 0.06$).

Normal tissues adjacent to aggressive vs. non-aggressive tumors. Normal tissues adjacent to aggressive tumors exhibited an upregulation of miR-107 and let-7f-5p ($P < 0.05$); the difference for miR-107 remained significant after FDR correction. Normal tissues adjacent to non-aggressive tumors displayed a downregulation of miR-34a-5p ($P < 0.05$).

NanoString counts were validated by qPCR (Fig. 2). A total of 4 samples were tested for let-7c expression and

4 were evaluated for miR-29c expression. A correlation of 0.93 was observed.

Discussion

The rising incidence of oral tongue SCC among young patients, combined with its weak association with known risk factors of other head and neck cancers (including smoking, ethylism and human papilloma virus infection) and its aggressiveness, have prompted researchers to seek molecular aberrations specific to the tumor in this age group (2,4-7). A high incidence of DNA ploidy abnormalities and microsatellite instability have been reported in young patients with tongue carcinoma (34,35). Molecular markers observed to be similar in young and old patients include p53, p21, retinoblastoma protein and mouse double minute 2 homolog expression; presence of mismatch repair genes such as human mutL homolog 1 and human mutS homolog 2; loss of heterozygosity at the 3, 9 and 17p regions; and high cell proliferation and angiogenesis index (34,36-39).

Numerous studies have reported that miRNA expression differs between cancer cells and healthy cells (22,28,40-45), although the exact mechanism by which each miRNA affects carcinogenesis is unclear. The present study sought, for the first time, to examine miRNA expression in oral cavity SCC in patients <30 years old. Comparisons of the tumor tissue with adjacent normal tissue revealed a significant upregulation of

let-7f in tumor tissues, which remained significant after FDR correction. This increase was attributed mainly to overexpression of let-7f in non-aggressive tumors and overexpression of let-7e in aggressive tumors compared with normal tissues.

The tumoral upregulation of let-7f is in line with previous reports in older patients. Christensen *et al.* (46) and Hui *et al.* (40) demonstrated overexpression of let-7f in oropharyngeal cancer compared with oral cavity cancer and benign tonsillar tissue, and Cui *et al.* (28) demonstrated overexpression of let-7f in oral cavity cancer compared with normal tissue. Tran *et al.* (23) reported similar results in cell lines of head and neck SCC.

Given the aggressiveness of recurrent oral cavity cancer in young patients with a high mortality rate and increased risk of distant metastases (14), the present study analyzed molecular markers separately in aggressive and non-aggressive tumors. The results revealed an overexpression of let-7e in aggressive tumors compared with adjacent normal tissues (paired analysis) that was not evident in non-aggressive tumors. No dysregulation of let-7e was observed in any of the other studies of oral cavity cancer or other head and neck cancers, possibly suggesting a unique biology of aggressive tongue SCC in young patients.

A second (non-paired) evaluation of aggressive vs. non-aggressive tumors demonstrated an upregulation of let-7c, let-7d-5p, miR-99a-5p, miR-29c-3p, miR-130a-3p and miR-361-5p in the aggressive tumors, with let-7c remaining significant after FDR correction. Previous reports of miRNA dysregulation in oral cavity cancer that used normal tissue as the control demonstrated no aberration in let-7c and downregulation of let-7 d in tumors (28,41-43). By contrast, in the present study, both markers were upregulated. Additionally, our findings for mir-99 differed from a previous study, in which forced expression of miR-99 family members in head and neck SCC cell lines led to a reduction in cell proliferation and cell migration, and an increase in apoptosis (47). In addition, mir-29 was downregulated in that study (47) and in another study of nasopharyngeal carcinoma associated with miR-29c expression with increased sensitivity to cisplatin and inhibition of invasion and metastatic spread (48), in contrast to the present findings.

The literature on the role of miR-130a is inconsistent. Studies of ovarian and hepatocellular carcinoma tumors and head and neck SCC cell lines reported an association between miR-130a downregulation with reduced response/resistance to cisplatin (49) and docetaxel (50), respectively, and conversely, an association between miR-130a upregulation and increased cisplatin resistance (51). In liver cancer, downregulation of miR-130a was associated with poor prognosis (52). However, studies of cervical and esophageal cancer noticed that miR-130a upregulation was associated with tumor development and poor prognosis (49,53), in line with our results in tumor vs. healthy cells. Similarly, in a study of 107 samples of oral cavity SCC, the copy number of mir-30b was frequently increased in advanced tumors, in correlation with hsa-miR-30b overexpression (54).

Previous studies evaluating let-7 expression in head and neck malignancies are summarized in Table II. The let-7 family of miRNAs are commonly considered tumor suppressors. They were originally discovered in the roundworm

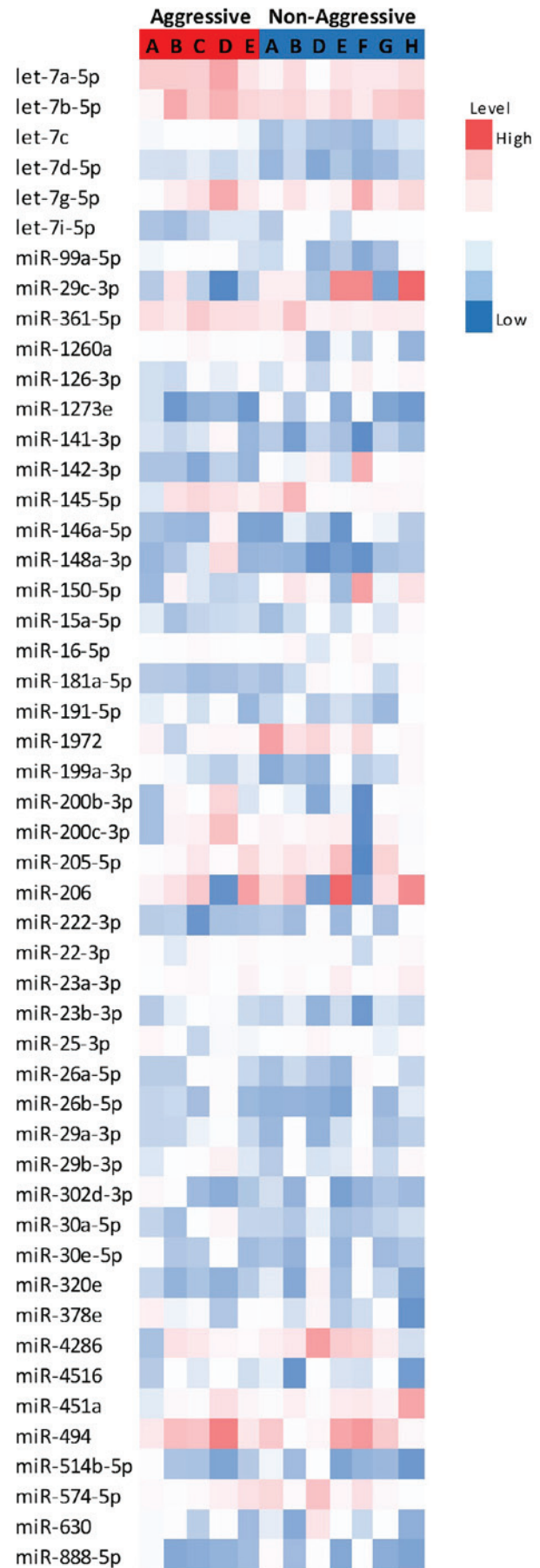


Figure 1. Heatmap of differentially expressed microRNAs (Z-scores of \log_2 values) in aggressive and non-aggressive tumors. Red indicates high expression and blue low expression. miR/miRNA, microRNA; hsa, *Homo sapiens*.

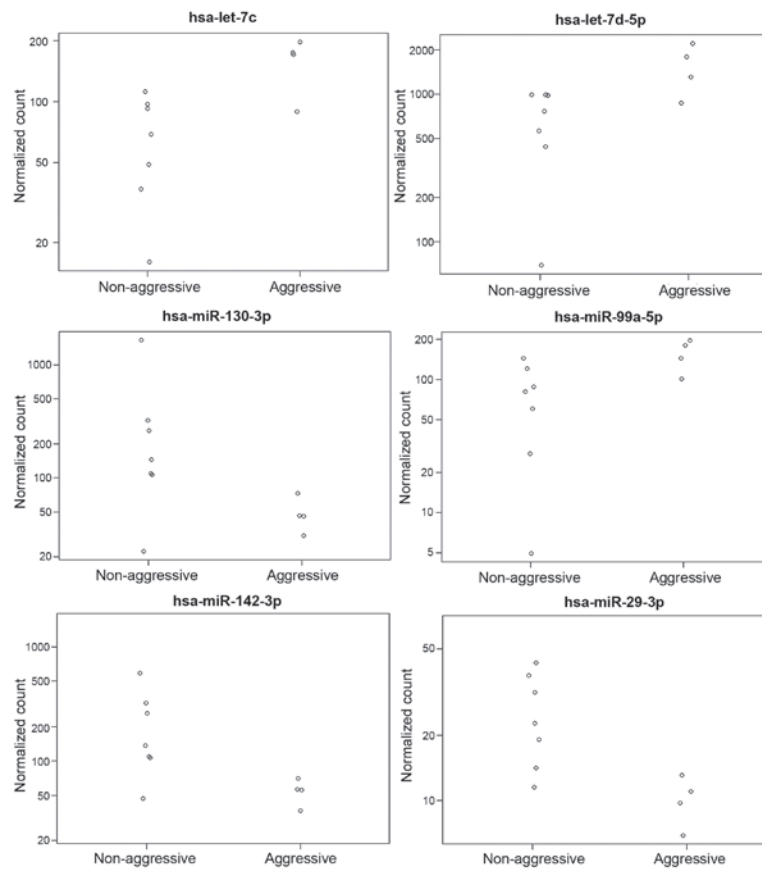


Figure 2. Plot of normalized counts for 6 microRNAs in 5 aggressive and 7 non-aggressive tumors. miR, microRNA.

Caenorhabditis elegans, a popular model in biology, where they were observed to be responsible for the development to the adult stage (55). let-7 miRNAs are dysregulated in lung, colorectal and gastric cancers (56-58). In lung cancer, reduced expression of let-7a was significantly associated with shortened postoperative survival, while its overexpression was significantly associated with inhibition of cancer cell growth (59). let-7 expression has also been associated with disease severity and survival in patients with head and neck cancer (56,60).

Jakymiw *et al* (44) hypothesized that the let-7 family affects cancer biology by regulating the expression of Dicer, an RNase III-type enzyme present in almost all eukaryotes. Dicer is essential for RNA interference and miRNA pathway maturation (61). Dicer was observed to be overexpressed in prostate and lung adenocarcinomas (62,63) and cervical cancers (53). Its overexpression (by 24-fold) in cell lines of head and neck cancer was associated with significantly reduced levels of let-7b. When the cancer cell lines were transfected with chemically synthesized let-7b, cell proliferation was inhibited by $\leq 83\%$ (44).

Findings of reduced let-7 levels in the presence of increased expression of the RAS protein (59) have led to the hypothesis that let-7 may inhibit cancer growth via its effect on the RAS protein. Accordingly, in head and neck SCC, the presence of the RAS protein was significantly associated with poor prognosis and increased tumor size (46,64). The inhibitory effect of let-7a in cancer development was further supported by findings of decreased let-7a levels in head and neck cancer and lymph node metastases (45).

Previous studies were inconsistent regarding the expression profiles of let-7a, let-7b, let-7c, let-7d and let-7f, with certain studies reporting overexpression while others reporting reduced expression in cancer (22-23,28,40-46). While the reduced expression of these miRNAs has provided a theoretical explanation to the mechanism by which cancer cells affect the RAS gene and protein, Dicer and HNC-TICS, no evidence has supported the upregulation of the let-7 family, which may explain the observed cell proliferation in head and neck cancers.

The majority of relevant studies conducted to date made no reference to patient age as a risk/contributory factor. Yu *et al* (45) compared miRNA expression in patients under and over the age of 50 years, and observed no difference between the groups.

The present study is affected by certain limitations. Oral cavity cancer in patients younger than 30 years of age is very uncommon (1,2,4-7). Consequently, our samples are small and the statistical power is limited. The present study evaluated miRNA profiles in young patients and compared the results with published data, rather than comparing them directly with samples from older patients. Thus, methodological differences between the current study and previous studies may affect our assumptions.

To conclude, the current study presented the miRNA profile of oral cavity SCC in patients younger than 30 years of age. Our finding of overexpression of let-7f in oral cavity cancer in young patients compared with adjacent normal tissue is in line with previous studies in older patients. However, further

Table II. let-7 dysregulation in head and neck carcinoma.

Study, author (ref)	Year	Collected samples	Tissue examined	n	Control	n	let-7	Expression	Age, years ^a
Chang <i>et al</i> (22)	2008	Primary tumors	HNSCC	4	Healthy tissue (UPPP patients)	4	let-7i	Upregulated	54
Tran <i>et al</i> (23)	2010	Cell lines	HNSCC	9	NA		let-7a	Upregulated	NA
							let-7b	Upregulated	
							let-7c	Upregulated	
							let-7d	Upregulated	
							let-7f	Upregulated	
Cui <i>et al</i> (28)	2014	NA	OCSCC	17	Healthy controls	3	let-7a	Upregulated	NA
							let-7f	Upregulated	
Christensen <i>et al</i> (46)	2009	Whole blood and buccal cells	OCSCC	10	Pharyngeal cancer	4	let-7a	Downregulated	NA
							let-7c	Downregulated	
							let-7d	Downregulated	
							let-7f	Downregulated	
Hui <i>et al</i> (40)	2013	FFPE	Oropharyngeal SCC	88	Tonsillectomy	7	let-7f	Upregulated	55±11
							let-7g	Upregulated	
Ricieri Brito <i>et al</i> (41)	2010	Primary tumors and whole blood	OCSCC	20	Self-control-healthy tissue		let-7a	Downregulated (n=18)	57 (37-90)
							let-7a	Upregulated (n=13)	
Chang <i>et al</i> (42)	2011	Cell lines	OCSCC	NA	Human normal keratinocyte cells	NA	let-7d	Downregulated	NA
MacLellan <i>et al</i> (43)	2012	Serum samples	CIS+OCSCC	30	Non-cancer patients serum	26	let-7a	Upregulated	50-93
							let-7b	Upregulated	
							let-7-d	Downregulated	
Jakymiw <i>et al</i> (44)	2010	Cell lines	HNSCC	6	Healthy gingival cells	NA	let-7b	Downregulated	NA
Yu <i>et al</i> (45)	2011	Primary tumors	Head and neck cancer	107	Self-control-healthy tissue		let-7a	Downregulated	NA
			metastases	50	No metastases	57	let-7a	Downregulated	
Current study	2015	Primary tumors	OCSCC	12	Self-control-healthy tissue	12	let-7f	Upregulated	15-30
			Aggressive OCSCC	5	Non aggressive OCSCC	7	let-7e	Upregulated	
							let-7c	Upregulated	
							let-7d	Upregulated	

^aAll ages are ranges, with the exception of Hui *et al.*, which is the mean age. CIS, carcinoma *in situ*; FFPE, formalin-fixed paraffin-embedded; HNSCC, head and neck squamous cell carcinoma; NA, not available; OCSCC, oral cavity squamous cell carcinoma; SCC, squamous cell carcinoma; UPPP, uvulopalatopharyngoplasty.

stratification of the tumors by aggressiveness revealed that let-7f was overexpressed in non-aggressive tumors, whereas the aggressive tumors had a distinctive miRNA expression pattern, with upregulation of let-7e (compared with normal tissue) as well as let-7c and let-7d (compared with non-aggressive tumors).

These findings may aid the prognosis of oral cavity tumors in young patients, which may be very aggressive and exhibit a high mortality rate. Further studies with direct comparison with tissues from older patients are warranted to validate the prognostic role of these miRNA aberrations in oral cavity SCC.

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