Salivary miR-93 and miR-200a as post-radiotherapy biomarkers in head and neck squamous cell carcinoma

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Abstract. Head and neck squamous cell carcinoma is the 6th most malignant tumor entity worldwide and has exhibited a 5-year mortality of approximately 50% for the last fifty years. For the therapy monitoring and successful management of this tumor entity new and easily accessible biomarkers are greatly needed. The aim of the study was to determine whether and to what extent microRNAs, a class of small regulatory RNAs, are detectable in saliva post-radiation therapy. The expression and feasibility as therapy monitoring marker of the microRNAs were analyzed by RT-qPCR in 83 saliva samples from 33 patients collected at several time points pre-, during and post-radiotherapy treatment. Ten head and neck squamous cell carcinoma- or radiation-associated microRNAs (miR-93, miR-125a, miR-142-3p, miR-200a, miR-203, miR-213, let-7a, let-7b, let-7g and let-7i) were analyzed. All were detectable to a different extent in the saliva of the patients. miR-93 and miR-200a were significantly higher expressed 12 months post-radiotherapy than at baseline (p=0.047 and p=0.036). These results point towards miR-93 and miR-200a as biomarkers for the treatment monitoring post-radiation of head and neck squamous cell carcinoma.

Introduction

Head and neck cancer is the term given to a variety of malignant tumors that develop in the oral cavity, laryngeal, pharynx and salivary glands. They are predominantly squamous cell carcinomas. Head and neck squamous cell carcinoma (HNSCC) is the 6th most common malignancy worldwide (1). The incidence rates in the world are higher in the most developed countries (2). It was estimated that there were approximately 550,000 new head and neck cancer cases in the world in 2008. The expected number of deaths was approximately 300,000. Despite progress in therapeutic procedures, patients with HNSCC have a high risk of early locoregional relapse. The five-year survival rate for HNSCC has shown only modest improvement over the last decades (3,4). Most of them are oral squamous cell carcinomas (OSCC) (5). Not only the delayed detection but also the difficulties to monitor the treatment and outcome are responsible for the high morbidity rate of HNSCC. Therefore, early detection of disease progression remains a challenging task mainly due to the lack of adequate biomarkers.

A variety of promising biomarkers has been described, nearly all of them can be detected immunohistochemically (6). In addition, saliva is preferentially studied for such biomarkers, because it has many advantages in terms of collection, storage, shipping, and voluminous sampling; all of these processes can be carried out very economically compared with serum or urine. All investigations focus on an early detection of OSCC (7). Moreover, salivary biomarkers are of great value for differentiation of an OSCC from potentially malignant oral disorders (8). However, the main advantages of saliva as a diagnostic tool for HNSCC is that oral cancer cells are immersed in the salivary milieu. Therefore saliva provides more direct information regarding the disease status. Saliva bears different biological components potentially capable for diagnosis and monitoring of HNSCC including the small nucleic acids designated as microRNAs (9). Several studies indicated the potential of saliva miRNAs for early oral cancer diagnosis (10-13).

MicroRNAs (miRNAs) are small (18-25 bp) non-coding RNAs of endogenous origin, which regulate gene expression post-transcriptionally by translation inhibition and increased decay of their target mRNAs. They recognize their target mRNA by binding of 6 nucleotide sequences in the 3'-UTR ('seed' sequence), which is specific for each individual microRNA species. To date, 2,588 individual microRNA sequences have been identified in human (miRBase database, release 21:

June 2014), which are estimated to regulate the expression of 60% of human protein-coding genes (14). MicroRNAs are essential regulators of cell proliferation, differentiation and apoptosis (reviewed in refs. 15-17). MiRNAs were recently shown to be abundant and stable in different body fluids such as serum, urine or saliva (18-20). Furthermore, altered expression of several microRNAs has already been described to be associated with the genesis and/or progression of oral cancer.

Among others, miR-93, miR-125a, miR-142-3p, miR-200a, miR-203, miR-213 (designated by actual miRBase terminology as miR-181a-3p), were found to be associated with HNSCC. miR-93, miR-125a, miR-142-3p and miR-200a were concordantly expressed in saliva of 12 HNSCC patients and 12 healthy controls (21). Furthermore, miR-125a and miR-200a expression was significantly lower in the saliva of a cohort of 50 HNSCC patients than in corresponding healthy controls (21). MicroRNA miR-203 was significantly downregulated in 23 samples of oral cavity SCC compared to control tissue (22). MicroRNA miR-213 was upregulated >3.0-fold in patients samples of squamous cell carcinoma of the tongue (23). Furthermore, miR-213 targets Nanog mRNA in a subpopulation of CD34$^+$ cells in peripheral blood, driving differentiation of components of the immune system (24). Despite their global role in oncogenic cell transformation members of the let-7 family (let-7a, let-7b, let-7g and let-7i) have been shown to be associated with radiation sensitivity and therefore may monitor radiotherapy in HNSCC (25). In detail, let-7a and -7b were downregulated by radiation, while let-7g and let-7i were upregulated and inhibition of let-7g by antagomirs led to increased radiosensitivity of lung carcinoma cells (26).

Radical surgical resection added with radiation and chemotherapy is the established curative treatment for HNSCC (27-29). The post-operative radiotherapy is intended to improve loco-regional control and therefore it is inevitable. Unfortunately, hyposalivation is the most common oral complication of radiation therapy (RT) for head and neck cancer patients (30). In addition to decreased saliva volume, RT also causes changes in saliva consistency, buffering capacity, and pH-value (31). However, in recent years several improvements, such as three-dimensional (3D) conformal radiation therapy (3D-CRT) and intensity-modulated radiation therapy (IMRT) have proven to be capable of sparing the salivary glands and thereby to prevent hyposalivation (32).

Hence, it is still unknown whether and to which extent microRNAs associated with HNSCC and radiosensitivity are detectable in oral fluid during and after radiotherapy and if they may act as salivary biomarkers for tumor monitoring. In this study, we screened a HNSCC- and radiation-associated panel of microRNAs for their feasibility as indicators of a tumor-free state of the oral cavity. It was the aim of the present investigation to analyze the prognostic impact of salivary microRNA levels of miR-93, miR-125a, miR-142-3p, miR-200a, miR-203, miR-213, let-7a, let-7b, let-7g and let-7i in patients receiving 3D-CRT treatment.

Materials and methods

Study population. Saliva samples were collected between 06/2002 and 10/2008 within the scope of our previously described study, wherein we examined the parotid gland

Table I. Patient demographics and tumor statistics.

Subject	Gender	Age (yrs.)	Tumor site	Stage		
1	Female	69	Oral, scc	T1	N0	Mx
2	Male	63	Laryngeal, scc	T3	N0	M0
3	Male	68	Oral, scc	T3	N0	M0
4	Male	56	Laryngeal, scc	T2	N2a	M0
5	Male	48	Oropharynegeal, scc	T3	N1	M0
6	Male	55	Laryngeal, scc	T2	N0	M0
7	Male	63	Laryngeal, scc	T1	N1	M0
8	Female	51	Oral, scc	T2	N0	M0
9	Male	40	Oral, scc	T2	N0	Mx
10	Male	38	Oral, scc	T1	N1	M0
11	Male	58	Oropharynegeal, scc	T2	N2b	M0
12	Male	78	Laryngeal, scc	T2	N2a	M0
13	Male	54	Oropharynegeal, scc	T3	N0	M0
14	Male	64	Laryngeal, scc	T3	N2c	M0
15	Female	47	Laryngeal, scc	T2	N2b	M0
16	Male	57	Oropharynegeal, scc	T2	N2b	M0
17	Male	79	Laryngeal, scc	T2	N1	M0

recovery after radiotherapy (33). The experimental protocol, consent form, and the present retrospective analysis of the saliva samples were approved by the intern institutional review board, the ethics committee of the Martin-Luther-University Halle-Wittenberg, in accordance to the Helsinki declaration. All patients gave written detailed informed consent.

Treatment planning and determination of the parotid gland doses. All patients received 3D-CRT or IMRT treatment planning as previously described (33). Patients were immobilized with individual thermoplastic head-neck-shoulder masks. The planning goal was, while maintaining a homogeneous dose distribution in the target volumes, to minimize mean dose in the contra-lateral parotid gland. No effort was undertaken to spare the submandibular, the sublingual or minor salivary glands. All patients were treated with continuous conventional fractionation and received 2.0-Gy fractions, 1 fraction per day, 5 fractions per week, for 7 weeks. The mean dose and the partial volumes receiving specified doses were determined for each gland from dose-volume histogram (DVH). Based on an algorithm initially proposed by Lyman the DVHs were transformed to single step DVHs (34). Afterwards, mean doses of the ipsilateral and contralateral parotid glands were calculated for every patient in Gy.

All patients underwent saliva collection at different time points: within one week before radiation treatment (baseline), during treatment and 6 and 12 months after the end of RT. All salivary samples were collected at least one hour after a meal at a standardized time of the day (9:00 am to 11:00 pm). Patients were asked to rinse the mouth and swallow any residual saliva. Then, the patients were instructed to chew on a paraffin pellet (Ivoclar Vivadent, Schaan, Liechtenstein) for 5 min. After 5 min samples were collected with the patients

expectorating all saliva into sterile test tubes. The samples were cryopreserved immediately after collection at -80˚C.

RNA isolation. Saliva (200 µl) was thawed to room temperature and immediately centrifuged (2000 x g, 10 min) to remove remaining cells and cellular debris. RNA was isolated by phenol/chloroform extraction using TRIzol (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Briefly, saliva supernatant was mixed with 750 µl of TRIzol reagent and 200 µl of chloroform. After centrifugation, the aequous phase containing the RNA was separated and remaining DNA was digested by 5 units DNase (Qiagen, Hilden, Germany). RNA was precipitated with 500 µl of ethanol overnight and washed several times with ethanol (96% and 70%). RNA concentration was analyzed after elution in 50 µl RNase-free H_2O using spectrometry (Eppendorf, Hamburg, Germany).

MicroRNA-specific cDNA synthesis. In this study, saliva expression of the following microRNAs was analyzed using TaqMan microRNA primer kits (Applied Biosystems, Darmstadt, Germany): miR-93, miR-125a, miR-142-3p, miR-200a, miR-203, miR-213, let-7a, let-7b, let-7g, let-7i and U18 snoRNA as reference gene. RNA (10 ng) were applied for each microRNA cDNA synthesis using specific RT primer (Applied Biosystems). RNA was incubated with RNAse Inhibitor, Buffer, dNTPs (20 mM) and MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) at 16˚C for 30 min and 42˚C for 30 min. Afterwards, reverse transcriptase was inactivated by a 5 min 85˚C step. cDNA was stored at -20˚C upon quantitative real-time analysis.

RT-qPCR analyses. cDNA of each individual patient sample was measured in RT-qPCR on a RotorGene 6000 (LTF Labortechnik, Wasserburg, Germany). For the reaction mix, buffer, dNTPS (20 mM), HotStartTaq-Polymerase (Qiagen) and microRNA-specific TaqMan primer (Applied Biosystems) were applied. Product accumulation was detected in fluorescence increase per cycle and quantified by ΔΔCq method according to Livak and Schmittgen (35). U18 snoRNA expression served as reference gene.

Statistical evaluation. Data were analyzed using SPSS software version 20 (IBM Inc., Chicago, IL, USA). Differences in microRNA expression between the patients groups were visualized by box-plots and analyzed by non-parametric tests (Mann-Whitney U test, Kruskal-Wallis test), and bivariate correlation analyses according to Spearman-Rho. P-values <0.05 were considered statistically significant.

Results

Longitudinal saliva samples were available for 17 patients: 3 women and 14 men (42 samples, termed Coh 1). Overall mean age was 57.8 years (range: 38-79 years). There were 4 patients with oropharyngeal squamous cell carcinomas, 5 patients with oral squamous cell carcinoma, and 8 patients with laryngeal squamous cell carcinoma. The majority were advanced tumors with regional node involvement at the time of treatment. None had distant metastases, whereas it was unknown in two
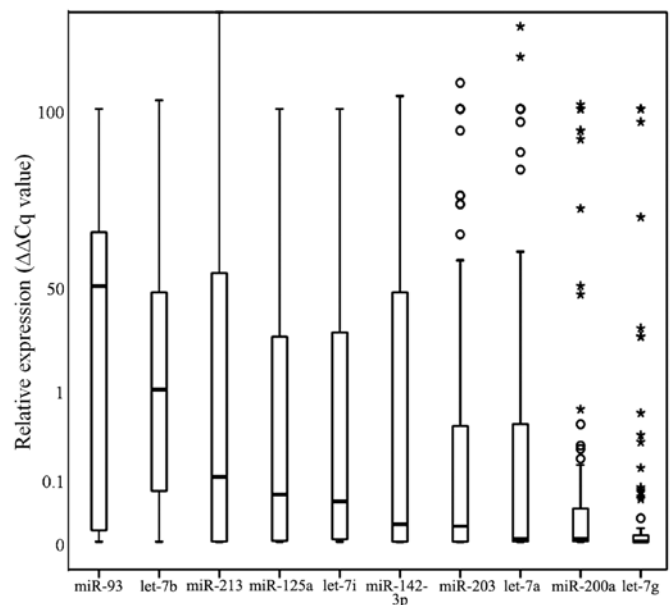

Figure 1. Variation of microRNA expression pattern in saliva. MicroRNAs are sorted by their median expression in a descending order. ΔΔCq values were analyzed, where expression of U18 snoRNA served as reference gene. Sample with lowest C_T value was chosen as calibrator. Y-axis was transformed to logarithmic for a better comparability of the different microRNA expression variances.

subjects (Table I). On average, patients received a mean dose of 27.7 Gy of the contralateral parotid gland.

Additional saliva samples at non-fixed intervals were collected from 16 patients: 3 women and 13 men (41 samples, termed Coh 0), which were analysed only for the determination of the effects of cryopreservation on the stability of microRNAs.

Measurement of saliva microRNAs. We were able to isolate RNA in detectable amounts from every saliva sample analyzed (Coh 0 + 1). In 95% of the samples analyzed, RNA concentrations ranged from 50 to 180 ng/µl. Expression levels of the selected microRNAs were measurable in the saliva samples to a different extent. miR-93 expression could be detected in 79 samples (95.2%), miR-125a in 71 samples (85.5%), miR-142-3p in 70 samples (84.3%), miR-200a in 80 samples (96.4%), mir-203 in 67 samples (80.7%), miR-213 in 73 samples (88.0%), let-7a in 78 samples (94.0%), let-7b in 68 samples (81.9%), let-7g in 56 samples (67.5%) and let-7i in 80 samples (96.4%). U18 snoRNA was detectable in each saliva sample and was used as reference gene.

MicroRNA expression between the single species was significantly different, ranging from a median relative expression of 58.3 (ΔΔCq value) for miR-93 to a median relative expression of 0.0005 (ΔΔCq value) for let-7g. The specific median relative expression values were as follows: miR-93: 0.583, let-7b: 0.103, miR-213: 0.018, miR-125a: 0.011, let-7i: 0.009, miR-142-3p: 0.0032, miR-203: 0.0028, miR-200a: 0.00004, let-7a: 0.00004, let-7g: 0.000005, respectively (Fig. 1).

In bivariate correlation analyses according to Spearman-Rho, especially miR-93 expression exhibited several highly significant associations to other microRNA

	Gender	Time post-radiotherapy	miR-93	miR-125a	miR-142-3p	miR-200a	miR-203	miR-213	let-7a	let-7b	let-7g	let-7i
Patient 1	male	6 months										
		12 months										
Patient 2	male	12 months										
Patient 3	female	6 months										
		12 months										
Patient 4	male	6 months										
Patient 5	male	6 months										
		12 months										
Patient 6	male	1 month										
		6 months										
Patient 7	male	12 months										
Patient 8	male	6 months										
Patient 9	female	6 months										
Patient 10	male	6 months										
Patient 11	female	6 months										
Patient 12	male	6 months										
		12 months										
Patient 13	male	6 months										
		12 months										
Patient 14	male	6 months										
Patient 15	male	1 month										
		12 months										
Patient 16	male	1 month										
		6 months										
Patient 17	male	12 months										

Figure 2. Comparison of microRNA expression pre- and post-radiotherapy. Saliva samples were collected at different time points after treatment. White fields depict >2.0-fold increase in relative expression, dark grey fields >2.0-fold decrease in expression. Grey fields depict a steady expression.

expression levels, namely miR-125a, miR-142-3p, miR-203, miR-213, let-7a and b (all positive correlations, Table II). On the other hand, miR-213 and let-7i expression levels as well as let-7a and let-7g expression levels were significantly negatively correlated (r_s= -0.31 and r_s= -0.39, p=0.005 and p=0.0003, respectively), indicating that there is no general microRNA overexpression in saliva of HNSCC patients.

MicroRNA expression pre- and post-radiotherapy. A subset of our cohort with comparable samples (Coh 1) was analyzed for distinct miRNA expression changes in our selected microRNA panel between baseline sample and post-treatment samples. MicroRNAs miR-142-3p (16 of 25 samples), miR-93 (15 of 25 samples) and miR-125a (15 of 25 samples) exhibited an increased level after radiotherapy, while microRNAs let-7a (17 of 25 samples) and let-7i (13 of 25 samples) showed a decrease in their expression after radiotherapy (Fig. 2). However, a consistently down- or up-regulated microRNA could not be identified in this first attempt.

In a more robust statistical encounter, we evaluated whether there is a significantly different expression of one or several microRNAs before (n=21), during (n=31) or after (n=31) radiotherapy without stratification according to the individual patients. In Mann-Whitney U tests, there was no significant alteration between during and post-treatment samples in the expression of the microRNAs tested (p-values ranging from p=0.14 for miR-200a and p=0.2 for miR-93 to p=0.97 for miR-203).

MicroRNA let-7i exhibited an increase in salivary expression during radiotherapy in comparison to the levels before radiotherapy (p=0.058, Mann-Whitney U test) and decreased

Table II. Bivariate correlation analyses according to Spearman-Rho.

miR correlations		r_s	p-value	n
miR-93 expression	miR-125a expression	0.34	0.002	83
	miR-142-3p expression	0.52	6.4×10^{-7}	83
	miR-203 expression	0.41	0.0001	83
	miR-213 expression	0.31	0.005	83
	let-7a expression	0.34	0.002	83
	let-7b expression	0.31	0.005	83
miR-125a expression	miR-213 expression	0.37	0.001	83
miR-213 expression	let-7a expression	0.41	0.0001	83
	let-7i expression	-0.31	0.005	83
let-7a expression	let-7g expression	-0.39	0.0003	83

miR-93 expression exhibited multiple positive correlations to several microRNAs. r_s, Spearman-Rank coefficient.

after treatment in comparison to the levels during radiotherapy (p=0.12, Mann-Whitney U test; Fig. 3A). However, Kruskal-Wallis analyses of the distribution of the specific microRNA expression between the phases pre-, during and post-radiotherapy revealed an attenuation for let-7i (p=0.87), whereas miR-200a remains unequally distributed over the different treatment phases and increases after radiotherapy (p=0.04, Fig. 3B).

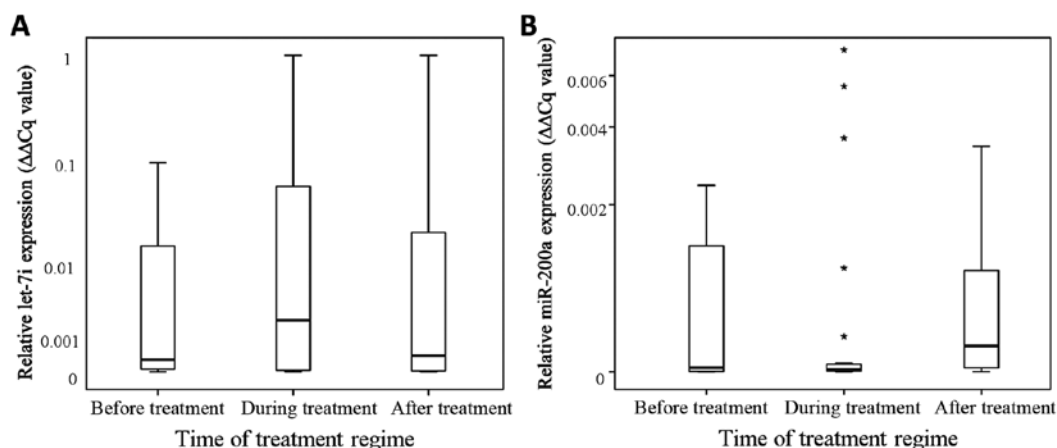Figure 3. let-7i (A) and miR-200a (B) microRNA expression distribution in saliva according to different phases of radiotherapy treatment. ΔΔCq values were analyzed, where expression of U18 snoRNA served as reference gene. Y-axis was transformed to logarithmic for a better comparability of the different microRNA expression variances. Note the different dilation of y-axis for let-7i and miR-200a, indicating the broader variance for let-7i in comparison to miR-200a.

Longitudinal distribution of microRNA expression post radiotherapy. Next, we evaluated the statistical distribution of relative expression levels of the selected microRNAs according to the individual patients with saliva samples from defined time points post-radiotherapy - baseline (n=17), 6 months post-therapy (n=14), and 12 months post-therapy (n=8). Of note, especially median miR-93 relative expression increases post-therapy in a time-dependent manner (Fig. 4). In Kruskal-Wallis analyses, no significant alterations of relative microRNA expression distribution were detected over the three time points (ranging from p=0.09 for miR-200a to p=0.98 for let-7i). Comparing miR-93 and miR-200a expression at baseline (n=17) with those at 12 months of post-therapy (n=8), both microRNAs were significantly higher expressed (p=0.047 and p=0.036 in Mann-Whitney U test, respectively).

MicroRNA expression in association to functional saliva gland parameter. Taking into account, that saliva production and secretion after radiotherapy is heavily dependent on saliva gland regeneration, we included the different saliva flow rates (in ml saliva/minute) of the patients at the different time points of collection (by calculating the ratio of microRNA expression/saliva flow rate) in our analyses. Noteworthy, in bivariate correlation according to Spearman-Rho miR-93 and let-7g expression were both associated to saliva flow rate (miR-93: r_s= -0.337; p=0.036; let-7g: r_s=0.334; p=0.037). The formerly detected associations between microRNA expression and time point of collection (baseline against 12 months post-treatment) remained after inclusion of saliva flow rates (p=0.049 for miR-93 and p=0.012 for miR-200a in Mann-Whitney U tests, respectively). However, in bivariate correlation analyses miR-93 expression was significantly positively correlated to miR-125a (r_s=0.59; p=0.000077), miR-142-3p (r_s=0.46; p=0.003) and let-7b (r_s=0.44; p=0.005), while miR-200a expression was only significantly correlated to let-7a expression (r_s=0.37; p=0.02). Furthermore, in a Kruskal-Wallis test the miR-200a expression/saliva flow ratio was significantly differently expressed at the end of treatment and 6 and 12 months after radiotherapy (p=0.036).

Discussion

The result of this study indicate that salivary microRNAs associated with HNSCC and radiosensitivity stay detectable in oral fluid during and after 3D-CRT. Therefore, salivary miRNA analysis still represents a potentially promising approach to monitor therapy response and recurrence of HNSCC in patients treated with radiotherapy. Firstly, we detected a significant increased expression of miR-200a comparing expression at baseline with that at 12 months post-radiotherapy. miR-200a is significantly less expressed in the saliva of OSCC patients in comparison to healthy controls and is considered as putative marker for tumor monitoring (21). Furthermore, low expression of miR-200a is associated with a worse outcome for ovarian carcinoma (36) and cervical cancer patients (37). In terms of pro-oncogenic action, decreased expression of miR-200a promotes the acceleration of meningioma cell growth (38) or the epithelial-mesenchymal transition and invasive potential of anaplastic thyroid carcinoma cells (39). miR-200a overexpression induced inhibition of nasopharyngeal carcinoma cell line growth via translation inhibition of ZEB2 and CTNNB1, while downregulation of miR-200a triggers the epithelial-mesenchymal transition of nasopharyngeal carcinoma cell lines also by ZEB2 activation (40). The increase of miR-200a detected in our cohort was significant in saliva samples at 12 months after end of treatment and may be a hint for wound healing processes and the return to a physiological state.

Furthermore, we detected a significant increased expression of miR-93. miR-93 is also significantly less expressed in the saliva of OSCC patients in comparison to healthy controls and it is a member of the miR-106b-25 cluster. miR-93 plays a role in cell proliferation and anchorage-independent cell growth in hepatocellular carcinoma by regulating for instance the transcription factor E2F1 (41) or the tumor suppressor gene FUS1 (42). In addition, miR-93 promotes tumor growth and angiogenesis in cocultured glioblastoma cell lines, pointing towards the oncogenic potential of the miR-106b-25 cluster (43). On the other hand, miR-93 overexpression inhibits proliferation and colony formation in colon cancer cells (44)
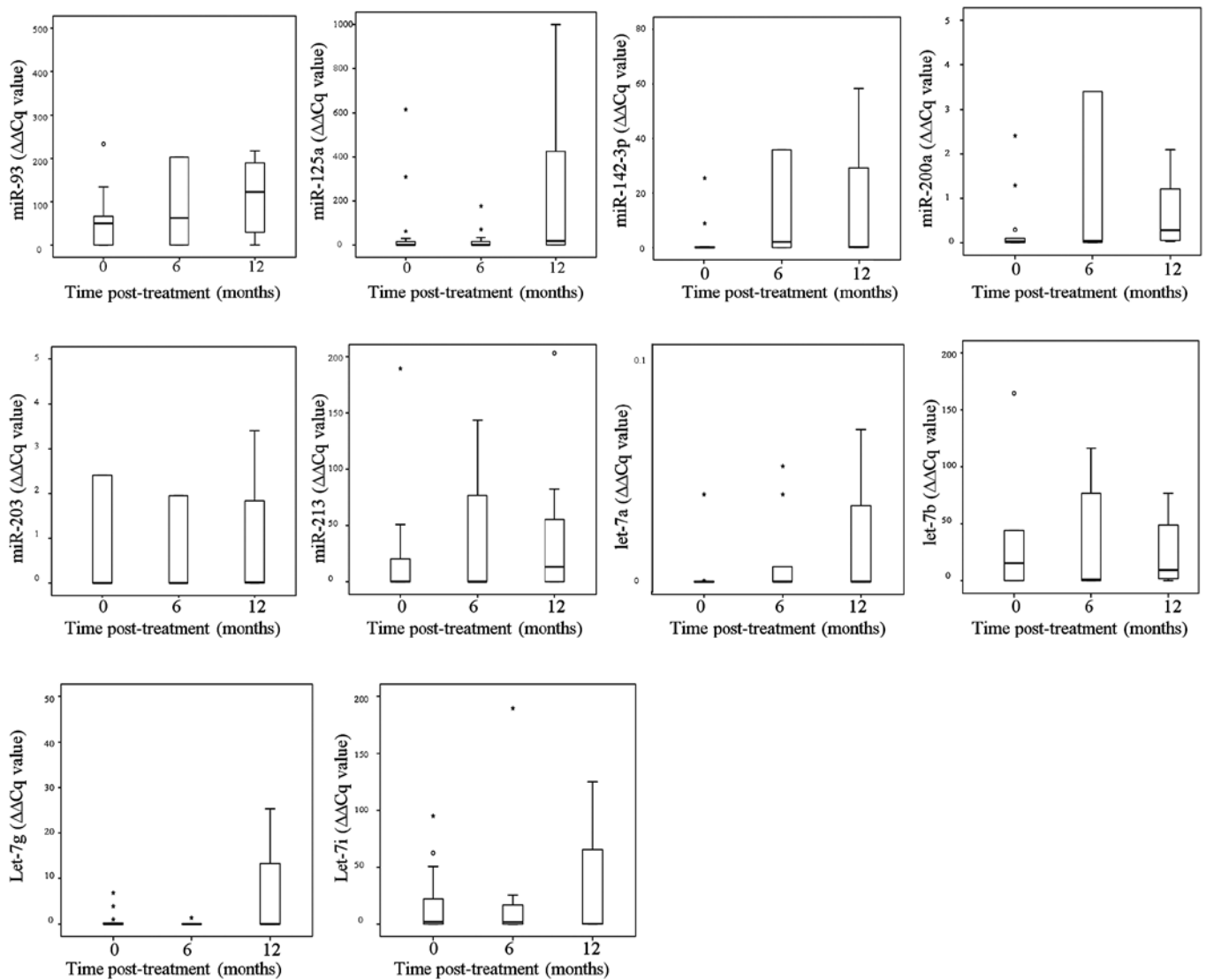
Figure 4. Longitudinal studies of relative microRNA expression according to 6 and 12 months post-radiotherapy in 17 individual patients. Baseline samples (n=17), 6 months post-radiotherapy samples (n=14) and 12 months post-radiotherapy samples (n=8) were analyzed by box-plot and Mann-Whitney U test. miR-93 and miR-200a median expression was significantly higher 12 months post-radiotherapy than at baseline (p=0.047 and p=0.036, respectively).

and acts differentially in several osteosarcoma cell lines (45). In gastric adenocarcinoma, elevated expression of miR-93 was detected and high miR-93 expression was significantly associated with an advanced disease stage and a decreased survival (46). Moreover, downregulated miR-93 expression was detected in gastric carcinoma, and low miR-93 expression was significantly correlated with an advanced tumor stage and an unfavorable outcome for patients (47). Altogether, these facts show a multi-faceted role of miR-93 in tumor genesis and progression. In our study, miR-93 re-induction and increased expression during post-treatment follow-up was demonstrated, a hint toward tumorsuppressive action of miR-93 in HNSCC progression.

let-7 family was demonstrated as clearly oncogenic transformation associated in several tumor entities such as lung cancer (48), gastric cancer (49) and breast cancer (50). The tumor suppressor roles of let-7 family in HNSCC were consistently demonstrated across studies. let-7 family is also a crucial modulator of stemness and HNSCC progression (51). In

this study, the let-7 family members were not clearly correlated with HNSCC treatment follow-up. Only let-7i showed a weak significant association with the application of ionizing radiation within the radiation therapy. However, this could be due to the diverging expression patterns and sometimes synonymous functions of this microRNA family.

These findings were limited by several factors. Most samples had a storage time over more than 5 years at -80°C and had not been stored by specialized protocols or additives. Extensive degradation of samples over time might be possible. However, in the light of several mostly forensic approaches it is likely that degradation was minimal. MicroRNA miR-15b, miR-16 and miR-24 have been proven to be stable in plasma at room temperature for at least 24 h, although introduction of a synthetic microRNA showed high RNAse activity (52). Furthermore, the analyzed microRNAs were still stable after samples underwent up to 5 freeze-thaw cycles (52). Stabilization of microRNA patterns in human whole saliva for up to two days at room temperature was achieved by

specialized protocols (53). Noteworthy, mRNA detection of saliva-specific marker in dried saliva stains stored at ambient temperature for up to 6 years and microRNA detection in saliva stains stored at the same conditions up to 1 year was possible in a forensic context (19,54).

Another limitation of the present study is the high standard deviations of the microRNA expression levels due to the small sample size. Thus, prospective analyses in an extended cohort are needed. Furthermore, the patients are only a subpopulation of patients from a previous study with a mean dose of 27.7 Gy of the contralateral parotid gland.

In conclusion, in this study we have shown for the first time that tumor monitoring with salivary microRNAs remains possible even in the radiation-related changes of saliva compounds. In addition to the results of Salazar and coworkers (55) we also developed a reliable method to isolate salivary miRNA at different times under radiotherapy of OSCC. Analysis of salivary microRNA expression in HNSCC patients after RT might potentially yield monitor markers, urgently asked for in the existing literature (56). Salivary miR-200a and miR-93 both were identified to be differentially expressed after radiotherapy and further validation of their role as well as other HNSCC-associated microRNAs such as miR-31 (57) in disease progression is warranted. Furthermore, larger studies about the potential of these microRNAs as tumor or therapy markers even in multicenter studies are possible, as we were able to show a stable detection of microRNA patterns after long-time storage at -80°C.

Acknowledgements

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