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HEAD & NECK

Expression levels of miR-34-family microRNAs are associated with *TP53* mutation status in head and neck squamous cell carcinoma

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Abstract

Purpose The majority of head and neck squamous cell carcinoma (HNSCC) cases in developing countries are associated with cigarette smoking and *TP53* mutations. p53 is a transcription factor that activates downstream genes, including the *hsa*-miR-34a and *hsa*-miR-34b/c loci, to achieve cell-cycle arrest, senescence, and/or apoptosis. This study examined the differences in expression levels of miR-34 in HNSCC with or without *TP53* mutations.

Methods We examined surgically resected tumor samples and normal adjacent tissues from HNSCC in oral cavity, larynx, and hypopharynx for *TP53* mutations (exons 5–8) and miR-34 expression levels.

Results miR-34a, miR-34b, miR-34b*, and miR-34c are significantly up-regulated in tumors with wild-type *TP53* genes ($n = 23$); while such up-regulation is not observed in tumors with mutant *TP53* ($n = 19$). Although expression levels of miR-34-family miRNAs do not correlate with gender, age, or tumor staging, interestingly they are correlated with smoking status and tumor sites. miR-34b/b*/c are up-regulated in tumors from those who ever smoked or recently smoked (quit smoking less than 15 years ago); but such up-regulation was not seen in those who never smoked or quit smoking for at least 15 years. HNSCC of the oral cavity also up-regulated miR-34b/b*/c while no such overexpression was observed in HNSCC of the larynx and hypopharynx.

Conclusions Surgically resected HNSCC samples with no *TP53* mutations have elevated levels of miR-34a and miR-34b/b*/c, while those with *TP53* mutations show no such up-regulation. miR-34b/b*/c expression is also correlated with smoking status and tumor sites.

Keywords Head and neck squamous cell carcinoma · HNSCC · MicroRNA · MiR-34 · *TP53*

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the cancer of squamous epithelial cells lining organs in the head and neck regions: oral cavity, larynx, hypopharynx, oropharynx, nasopharynx, paranasal sinuses, nasal cavity, and salivary glands. New cases of HNSCC were estimated to be approximately 5% of all cancer cases worldwide [1]. The main risk factor of HNSCC has been cigarette smoking, although in recent years increasing number of cases with tumors in the oropharynx in developed countries are associated with human papillomavirus (HPV) infection [2]. Additionally, nasopharyngeal carcinoma has been shown to be strongly associated with EBV infection [3].

In the group of HNSCC that is not associated with HPV, it has been demonstrated that the majority of them is associated with a history of cigarette smoking and *TP53* gene mutations [2]. p53 is a tumor suppressor protein that acts as a transcription factor in response to DNA damage and other stress signals [4]. p53 activation can lead to apoptosis or senescence; and thus defected p53 allows tumor cells to escape cell death or cell-cycle arrest. There are several proteins and non-coding RNAs downstream of p53, including miR-34-family miRNAs [5].

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microRNAs (miRNAs) are small non-coding RNAs (~ 19 to 22 nucleotides) that function generally in down-regulating their targets at the post-transcriptional level. Human microRNA-34 family includes several miRNAs: miR-34a (*hsa*-miR-34a-5p) and miR-34c (*hsa*-miR-34c-5p) are more predominantly detected mature forms of miR-34a and miR-34c primary transcripts, respectively; on the other hand, from the same primary transcript both miR-34b (*hsa*-miR-34b-3p) and miR-34b* (*hsa*-miR-34b-5p) are found at a similar level (miRBase Release 22, March 2018) [6]. In human, *hsa*-miR-34a is expressed from the locus on chromosome 1 and *hsa*-miR-34b, *hsa*-miR-34b*, and *hsa*-miR-34c are expressed as one transcription unit from chromosome 11 (Fig. 1a) [6–8]. Both miR-34 loci have p53-binding sites preceding them and have been demonstrated to be under direct activation of p53 [7]. p53-induced expression of miR-34a has been shown to promote apoptosis [9–11]; while p53-induced expression of miR-34b and miR-34c suppresses cell proliferation [12]. Yet, miR-34 loci are not only regulated by p53, but transcription from the miR-34a locus is also under the control of ELK1 [13] and FoxO3a also regulates transcription from the miR-34b/c locus [14].

In head and neck cancer, up-regulation of miR-34b has been recorded in several studies (as reviewed in [16]), but there has also been a report that miR-34b/c is down-regulated in nasopharyngeal cancer [17]. Since the activation of miR-34a and miR-34b/c transcription by p53 can obviously be disrupted by non-functional, mutated p53, several studies have also associated lower miR-34 expression with *TP53*-mutated cancers when compared to tumors with wild-type *TP53* gene, including miR-34a in chronic lymphocytic leukemia [18–21] and miR-34a, miR-34b*, and miR-34c in ovarian cancer [22].

In this study, we hypothesized that, similar to in leukemia and ovarian cancer, expression of miR-34-family miRNAs depend on the presence of functional p53. Since the majority of HNSCC with no HPV infection is associated with smoking and *TP53* mutations, expression of miR-34 is likely to vary in HNSCC depending on p53 mutation status. This study aims to determine whether *TP53*-mutated tumors fail to activate miR-34 expression in HNSCC tumor specimens, which would suggest that in this type of tumor, similar to in lung cancer or leukemia, exogenous addition of miR-34 may help mediate cell-cycle arrest and/or apoptosis function that is currently repressed.

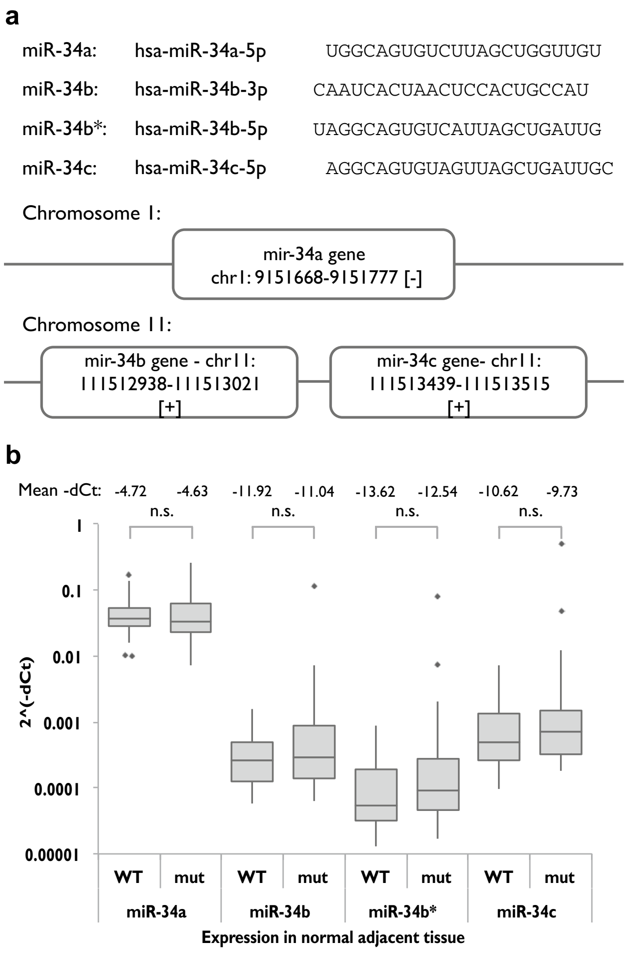


Fig. 1 miR-34 loci and expression levels of miR-34a, miR-34b, miR-34b*, and miR-34c in normal adjacent tissues. **a** Nomenclatures and sequences of miR-34-family miRNAs. **b** Expression levels of miR-34a, miR-34b, miR-34b*, and miR-34c in the groups with no *TP53* mutation ("WT"; $n = 23$) and with mutant *TP53* ("mut"; $n = 19$) are shown as $2^{-\mathrm{dCt}}$ values [15]. DeltaCt (dCt) values were calculated by subtracting Ct of RNU6B (control gene) from Ct of miRNAs indicated. Ct values were determined by Roche LightCycler® 480 qRT-PCR instrument. Mean − dCt in each group is shown above its box plot; lower number of − dCt indicates lower expression of miRNAs (e.g., $-11 < -4$). *n.s.* not statistically significant as analyzed by unpaired Student's *t* test

Materials and methods

Patients and samples

All patients were recruited from ENT clinic, Siriraj Hospital, Bangkok, Thailand. Patients recruited were previously diagnosed with head and neck squamous cell carcinoma in the larynx, hypopharynx, or oral cavity regions and were recommended for treatment by surgery. The study was then communicated to the patients before consent was obtained. Each patient was interviewed for their history relevant to the study, including smoking history, family history of tumor, and age. After surgery, resected tumor and adjacent tissues were sent for pathological diagnosis as part of routine clinical practice. After pathological analysis was completed, tumor samples and normal adjacent tissues (10 µm thick, ~ 150 to 300 mm^2) were then cut for miR-34 expression and p53 mutation analyses. This study has been approved by Siriraj Institutional Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University.

RNA and DNA extraction

RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) samples of tumors and normal adjacent tissues (NAT) with the miRNeasy FFPE Kit (Qiagen, cat. no. 217504) according to the manufacturer's protocol. DNA was extracted from tissues sliced from the same FFPE blocks with the QIAamp DNA FFPE Tissue Kit (Qiagen, cat. no. 56404). RNA and DNA concentration and purity were then measured by a nanophotometer (Implen Nanophotometer P300).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Reverse transcription was performed according to the protocol of miScript II RT Kit (Qiagen, cat. no. 218161). Real-time PCR was done in triplicates according to the protocol of miScript SYBR® Green PCR Kit (Qiagen, cat. no. 218073) using miScript Primer Assay as follows: Hs_RNU6-2_11, Hs_miR-34a_1, Hs_miR-34b_2, Hs_miR-34b*_2, and Hs_miR-34c_1 (Qiagen, cat. no. 218300). cDNA converted from 5 ng of RNA was used for each 10-µl PCR reaction in a 384-well plate (Roche, cat. no. 04729749001). qRT-PCR was performed on LightCycler® 480 Instrument (Roche, cat. no. 05015243001), which is set to determine Ct value for each well—these numbers were then used in the calculation of expression levels.

Polymerase chain reaction (PCR) and sequencing

All PCR reactions were done according to the manufacturer's protocol for AmpliTaq Gold 360 Master Mix (Thermo Fisher, cat. no. 4398881). Primers for HPV L1 region were degenerate primers MY11 and MY09 [23]; and primers for TP53 are shown in Table 1. 400 ng of genomic DNA was used for each reaction. Amplicons containing exons 5–8 of *TP53* were then sent for Sanger sequencing by Bioneer Sequencing Service (South Korea).

Results

Demographic and clinicopathological data of recruited patients and their resected tumors

Forty-two patients were recruited for this study (Table 2). Half of them ($n=21$, 50%) presented with HNSCC in the oral cavity, while the other half ($n=21$, 50%) presented with HNSCC in the larynx ($n=14$, 33.3%) or hypopharynx ($n=7$, 16.7%). We did not recruit any patients with tumor in the oropharynx, which is the region where human papilloma virus (HPV)-positive tumors were commonly found [2]; however, we still tested all tumor samples for HPV status by PCR amplification of the HPV L1 region [23]. As expected there was no HPV DNA present in any of tumor tissue samples. One of the exclusion criteria was insufficient DNA or RNA amount; for that reason, we excluded all stage I tumor samples due to their small size. The majority of tumors included in this study are at TNM stage IV ($n=20$, 47.6%), followed by stage III ($n=16$, 38.1%) and stage II ($n=6$, 14.3%).

As is common in HNSCC [2], the majority of the patients in this study were male ($n=33$, 78.6%) and had a history of smoking ($n=32$, 76.2%). Calculating from only those who ever smoked ($n=32$), their mean pack-year is 23.0 (range 0.5–68 pack years) and they quitted smoking 3.8 years ago on average (range 0–30 years). Mean age of recruited patients was 62.6 years (95% confidence interval = 59.1, 66.1) (Table 2).

TP53 mutation identification

Sites for mutations in *TP53* are commonly found in exons 5–8 [26–28]. Therefore, we amplified exons 5–8 region from tumor genomic DNA by PCR and sent them for direct sequencing. 19 tumor samples (45.2%) were found to have mutations in exons 5–8 of *TP53*, while 23 samples (54.8%)

Table 1 Primers for amplification of exons 5–8 of *TP53*

Target amplicons	Primer sequence
Exon 5 forward, exons 5–6 forward	CTGTTCACTTGTGCCCTGAC
Exon 5 reverse	CAATCAGTGAGGAATCAGAGGC [24]
Exon 6 forward	TCAGATAGCGATGGTGAGCAG [24]
Exon 6 reverse, exons 5–6 reverse	CCACTGACAACCACCCTTAAC [25]
Exon 7 forward, exons 7–8 forward	CGCACTGGCCTCATCTTGG
Exon 7 reverse	GAAATCGGTAAGAGGTGGGC [24]
Exon 8 forward	GGAGTAGATGGAGCCTGGTTT [24]
Exon 8 reverse, exons 7–8 reverse	GTGAATCTGAGGCATAACTGCA

Table 2 Demographic and clinicopathological data

	All patients ($n=42$)	No *TP53* mutation detected ($n=23$)	Mutations in *TP53* detected ($n=19$)	p value (WT vs. *TP53* mutation)
Gender				
Male	33 (78.6%)	17 (73.9%)	16 (84.2%)	0.4766 (Fisher's exact test)
Female	9 (21.4%)	6 (26.1%)	3 (15.8%)	
Age (years)				
Mean (range)	62.6 (22–83)	64.4 (46–83)	60.6 (22–80)	0.2843 (Student's t test)
95% CI	59.1, 66.1	60.2, 68.5	54.3, 66.9	
Smoking				
Smoker	32 (76.2%)	18 (78.3%)	14 (73.7%)	1.0000 (Fisher's exact test)
Non-smoker	10 (23.8%)	5 (21.7%)	5 (26.3%)	
Out of those who smoke				
Mean pack years ± SD (range)	23.0 ± 16.1 (0.5–68)	18.6 ± 12.3 (0.5–40)	28.7 ± 18.8 (7–68)	0.0787
Years since quitting ± SD (range)	3.8 ± 7.05 (0–30)	3.7 ± 6.4 (0–20)	3.9 ± 8.1 (0.1–30)	0.9597 (Student's t test)
HPV status				
HPV DNA present	0 (0.0%)	0 (0.0%)	0 (0.0%)	1.0000 (Fisher's exact test)
No HPV DNA present	42 (100.0%)	23 (100.0%)	19 (100.0%)	
Site				
Oral cavity (OC)	21 (50.0%)	14 (60.9%)	7 (36.8%)	OC vs. L/H
Larynx and hypopharynx (L/H)	21 (50.0%)	9 (39.1%)	12 (63.2%)	0.2146 (Fisher's exact test)
Larynx	14 (33.3%)	6 (26.1%)	8 (42.1%)	
Hypopharynx	7 (16.7%)	3 (13.0%)	4 (21.1%)	
Tumor staging				
I	0 (0.0%)	0 (0.0%)	0 (0.0%)	I/II vs. III/IV
II	6 (14.3%)	5 (21.7%)	1 (5.3%)	0.1973 (Fisher's exact test)
III	16 (38.1%)	9 (39.1%)	7 (36.8%)	
IV	20 (47.6%)	9 (39.1%)	11 (57.9%)	

did not have any mutations or had silent mutations (no change in amino acid sequence of P53 protein). There were no statistically significant differences in gender, age, smoking history, HPV status, and tumor site and staging between the groups with and without *TP53* mutations in exons 5–8 (Table 2).

Expression of miR-34-family microRNAs in normal adjacent tissues

MicroRNA-34 family includes several mature miRNAs: miR-34a (*hsa*-miR-34a-5p) transcribed from chromosome 1 and miR-34b (*hsa*-miR-34b-3p), miR-34b* (*hsa*-miR-34b-5p), and miR-34c (*hsa*-miR-34c-5p) from chromosome 11 (Fig. 1a) [6, 8]. qRT-PCR of mature miRNAs for all 4 of them and RNU6B, as a control, was performed for all tumor samples and normal adjacent tissues (NATs). As *TP53* mutation is likely to be somatic mutations, which are presented only in tumors but not in NATs, we expected to see no differences in the levels of expression of all four miR-34 microRNAs in normal adjacent tissues between groups with and without *TP53* mutations in their corresponding tumor samples. Indeed, no statistically significant differences were detected when we measured levels of expression of miR-34a, miR-34b, miR-34b*, and miR-34c in normal adjacent tissues and compared them between those two groups (Fig. 1b). In addition, this result also indicates that miR-34a was expressed at a higher level than miR-34b, miR-34b*, and miR-34c in non-tumorous squamous epithelial cells of the head and neck region.

miR-34-family microRNAs were up-regulated in tumors

Next, we compared the levels of expression of miR-34-family miRNAs in tumor samples with those in normal adjacent tissues to determine if tumors up-regulated miR-34-family miRNAs. First, we examined all samples ($n=42$), disregarding their *TP53* status, and found that miR-34a, miR-34b, and miR-34b* were expressed at significantly higher levels in tumors than in normal adjacent tissues on average (Fig. 2).

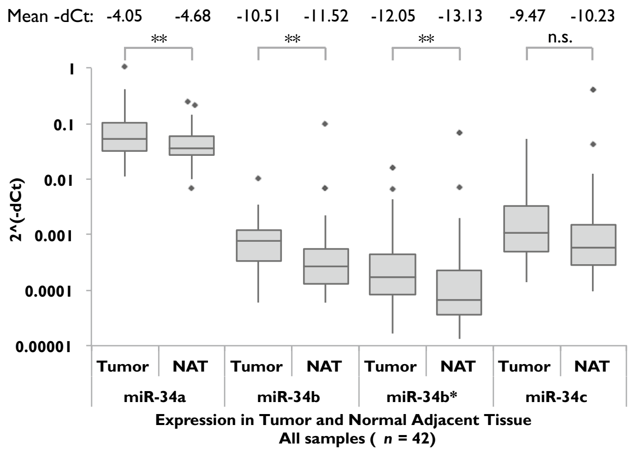


Fig. 2 Expression levels of miR-34a, miR-34b, and miR-34b* were significantly higher in tumors than in normal adjacent tissues. Expression of miR-34-family miRNAs shown as $2^{-\mathrm{dCt}}$ values, calculated as in Fig. 1 when all samples are examined together disregarding their *TP53* mutation status. Mean − dCt in each group is shown above its box plot; ***p* value < 0.01; and *n.s.* not statistically significant—as analyzed by paired *t* test

miR-34-family miRNAs were expressed at higher levels in tumor samples than in NATs in samples with wild-type *TP53*, but similar up-regulation was not observed in samples with *TP53* mutations

Second, we analyzed whether the up-regulation of miR-34a, miR-34b, and miR-34b* could only happen when *TP53* gene was intact. In analyzing miR-34 expression in the group with no *TP53* mutations detected (“WT *TP53*”) ($n = 23$), it was found that expression of miR-34a, miR-34b, miR-34b*, and also miR-34c in tumors increased with statistical significance when compared to expression in their matching NATs (Fig. 3a, c). However, when the group of samples with *TP53* mutations (“mut *TP53*”) ($n = 19$) was examined, it was found that those tumor samples did not significantly overexpress miR-34a, miR-34b, miR-34b*, and miR-34c when compared to their matching NATs (Fig. 3b, d).

We then compared the expression levels of miR-34-family miRNAs between tumors in the WT *TP53* group and tumors in the mut *TP53* group after each was normalized to their corresponding NATs. There was no statistically significant difference between them (Fig. 3e). This is likely to be because while samples in the WT *TP53* group significantly up-regulated miR-34-family miRNAs (Fig. 3a, c), several samples in the group with *TP53* mutations (mut *TP53*) expressed them at slightly higher levels than their corresponding NATs (Fig. 3b, d); therefore, the differences between the two groups may not be that large.

The association of expression levels of miR-34-family microRNAs and demographic and clinicopathological data

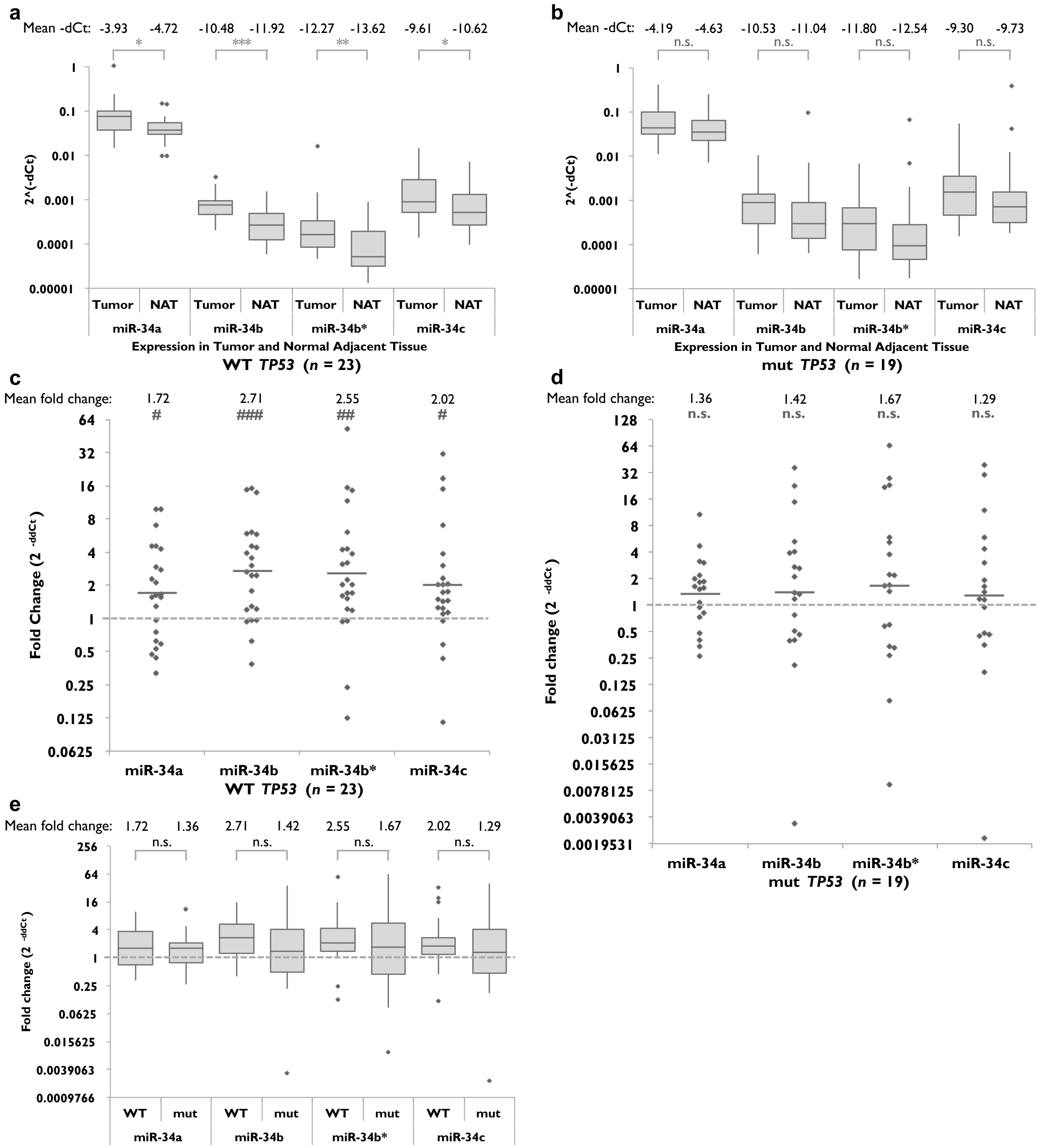
In addition to *TP53* mutations, we further examined the association between levels of expression of miR-34a, miR-34b, miR-34b*, and miR-34c with demographic and clinicopathological data of recruited patients (Table 3; Fig. 4a–f). Levels of expression of all four miR-34-family microRNAs are not associated with gender (male vs. female), the number of cigarettes consumed per day (0–10 vs. 11+ cigarettes per day), or stages of tumor (I/II vs. III/IV) (Table 3 and dCt data not shown).

Interestingly, there was an association between expression levels of miR-34b, miR-34b*, and miR-34c and two of the smoking parameters: whether they ever smoked (Fig. 4a, d) and the duration of time since they have quitted smoking (more or less than 15 years) (Fig. 4b, e). In similar patterns, tumors from patients who ever smoked ($n = 32$) or from those who smoked recently (quit less than 15 years before sample collection, $n = 25$) expressed significantly more miR-34b, miR-34b*, and miR-34c than their matching NATs. On the other hand, tumors from patients who never smoked ($n = 10$) or from those who never smoked or had stopped smoking for at least 15 years ($n = 17$) did not have elevated levels of expression of miR-34b, miR-34b*, and miR-34c in tumor samples when compared to their corresponding NATs (Fig. 4a, b, d, e).

Moreover, there was an association between expression levels of miR-34b, miR-34b*, and miR-34c and tumor site (oral cavity vs. larynx or hypopharynx) (Fig. 4c, f). Squamous cell carcinoma (SCC) in the oral cavity region expresses significantly more miR-34b, miR-34b*, and miR-34c than their matching NATs, while SCC in the larynx or hypopharynx did not significantly up-regulate these 3 miRNAs when compared to their matching NATs (Fig. 4c, f).

Discussion

In this study, we examined tumor tissue and matching normal adjacent tissue samples from 42 patients diagnosed with HNSCC in the larynx, hypopharynx, and oral cavity. Of these 42 tumor samples, 45.2% ($n = 19$) was found to have mutations in *TP53* gene. The rate of *TP53* mutation found in this study is lower than 72% found by The Cancer Genome Atlas consortium (TCGA) [2], but close to the frequency of 58% of samples showing strong p53 expression determined by another group in Thailand [29]. This suggests that the rate of *TP53* mutations could partly be due to ethnicity of subjects. As we excluded oropharyngeal cancer, which is the usual site of HPV-associated tumors, from this study, 0% ($n = 0$) had HPV DNA detected in the cells as expected.


a
Mean -dCt: -3.93 -4.72 -10.48 -11.92 -12.27 -13.62 -9.61 -10.62
* *** ** *
1
0.1
0.01
0.001
0.0001
0.00001
2^(-dCt)
Tumor NAT Tumor NAT Tumor NAT Tumor NAT
miR-34a miR-34b miR-34b* miR-34c
Expression in Tumor and Normal Adjacent Tissue
WT TP53 (n = 23)
b
Mean -dCt: -4.19 -4.63 -10.53 -11.04 -11.80 -12.54 -9.30 -9.73
n.s. n.s. n.s. n.s.
1
0.1
0.01
0.001
0.0001
0.00001
2^(-dCt)
Tumor NAT Tumor NAT Tumor NAT Tumor NAT
miR-34a miR-34b miR-34b* miR-34c
Expression in Tumor and Normal Adjacent Tissue
mut TP53 (n = 19)
c
Mean fold change: 1.72 2.71 2.55 2.02
#
64
32
16
8
4
2
1
0.5
0.25
0.125
0.0625
Fold Change (2 -ddCt)
miR-34a miR-34b miR-34b* miR-34c
WT TP53 (n = 23)
d
Mean fold change: 1.36 1.42 1.67 1.29
n.s. n.s. n.s. n.s.
128
64
32
16
8
4
2
1
0.5
0.25
0.125
0.0625
0.03125
0.015625
0.0078125
0.0039063
0.0019531
Fold change (2 -ddCt)
miR-34a miR-34b miR-34b* miR-34c
mut TP53 (n = 19)
e
Mean fold change: 1.72 1.36 2.71 1.42 2.55 1.67 2.02 1.29
n.s. n.s. n.s. n.s.
256
64
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0.0625
0.015625
0.0039063
0.0009766
Fold change (2 -ddCt)
WT mut WT mut WT mut WT mut
miR-34a miR-34b miR-34b* miR-34c

◂**Fig. 3** miR-34a, miR-34b, miR-34b*, and miR-34c were expressed at higher levels in tumor tissues than in NATs in the group with wild-type *TP53*, but similar up-regulation was not observed in the group with *TP53* mutations. **a, c** Tumor samples without *TP53* mutations (“WT *TP53*”); **b, d** samples with *TP53* mutations (“mut *TP53*”). **a, b** Expression of miR-34-family miRNAs as $2^{-\mathrm{dCt}}$ values, calculated as in Fig. 1. Mean − dCt in each group is shown above its box plot; $*p$ value < 0.05; $**p$ value < 0.01; $***p$ value < 0.0001; and n.s., not statistically significant—as analyzed by paired *t* test. **c, d** Expression of miR-34-family miRNAs as fold change [15] (mathematically: $2^{-\mathrm{ddCt}}$) in tumor samples after normalization by matching NATs. Delta-delta-Ct (ddCt) values were calculated by subtracting dCt of NAT from dCt of matched tumor samples. Short horizontal lines across dot plots indicate mean fold change for each miRNAs with the values of mean fold change written above. Long dashed lines indicate fold change = 1 which means no change in expression; $^{\#}p$ value < 0.05; $^{\#\#}p$ value < 0.01; $^{\#\#\#}p$ value < 0.0001; and *n.s.* not statistically significant—as analyzed by one-sample *t* test when the null hypothesis is no change in expression. **e** Expression of miR-34-family miRNAs in tumors with and without *TP53* mutations as fold change. Fold change is calculated as described above; mean fold change is indicated above the box plots. *n.s.* not statistically significant—as analyzed by unpaired Student’s *t* test

Furthermore, we showed that there was no difference in expression levels of miR-34a, miR-34b, miR-34b*, and miR-34c in normal adjacent tissues whether there is any mutations detected in *TP53* gene in the matching tumor tissues (Fig. 1b). Therefore, any differences in relative expression of miR-34-family miRNAs between those with and without *TP53* mutations are a result of miRNA expression levels in tumor tissues themselves. Overall, this study has demonstrated that miR-34a, miR-34b, and miR-34b* were up-regulated in HNSCC samples, disregarding their *TP53* mutation status, when compared to expression in their matching normal adjacent tissues (Fig. 2). This observation is consistent with previous studies: miR-34b has been shown to be up-regulated in HNSCC [16, 30–32]. On the contrary, there were other studies that show different observations, such as down-regulation of miR-34c in laryngeal SCC [33]; up-regulation of miR-34c in SCC of the tongue and HNSCC [30, 32]; and down-regulation of miR-34a in SCC of the tongue [34]. We hypothesized that miR-34a and miR-34b/b*/c expression depends in part on normal functions of p53 as p53 has been shown to be their transcriptional regulators [4, 7, 9, 35–37]. Therefore, expression of miR-34-family miRNAs in samples with and without *TP53* mutations may show different patterns.

For the group of tumor tissues with no mutations detected in *TP53* exons 5–8, which is the hotspot for mutations (“WT *TP53*”), expression levels of miR-34a, miR-34b, miR-34b*, and miR-34c are higher than those in their matching NATs (Fig. 3a, c). This is consistent with other studies that demonstrated that p53 protein could stimulate transcription of both the miR-34a locus on chromosome 1 and the transcription cluster of miRNAs that expresses miR-34b, miR-34b*, and miR-34c on chromosome 11 [4, 7, 9, 12, 35–37]. On the other hand, it is also consistent with our findings that in the group with mutations detected in *TP53* (“mut *TP53*”), expression levels of miR-34a, miR-34b, miR-34b*, and miR-34c did not significantly increase (Fig. 3b, d). Since p53 may not be fully functional in these samples due to mutations, it is reasonable to expect that miR-34a and miR-34b/b*/c loci did not experience a significant increase in transcription. Lower expression of miR-34-family miRNAs in *TP53*-mutated tumors when compared to tumors with WT *TP53* is also observed in other types of cancers, such as miR-34a in chronic lymphocytic leukemia [18–21] and miR-34a, miR-34b*, and miR-34c in ovarian cancer [22]. However, this is the first time that this has been observed in head and neck squamous cell carcinoma.

However, when expression levels of miR-34a, miR-34b, miR-34b*, and miR-34c in the groups with and without *TP53* mutations were compared to each other, the differences were not significant (Fig. 3e). This is likely to be due to the fact that the four miR-34-family miRNAs were slightly, though not significantly, up-regulated in tumors with *TP53* mutations as well. It is possible that the up-regulation of miR-34-family miRNAs observed in this study does not depend solely on p53 protein function, but instead their transcription may possibly be activated by other factors as well. This is possible, as previous studies have shown that miR-34a and the miR-34b/b*/c cluster loci are under the control of not only p53, but also ELK1 and FoxO3a, respectively [4, 13, 14].

In correlating miR-34-family miRNAs with demographic and clinicopathological data, this study demonstrates that the group of patients who ever smoked ($n = 32$) and who have smoked recently (quitting smoking less than 15 years ago; $n = 25$) had higher expression of miR-34b, miR-34b*, and miR-34c in the tumors than their matching normal adjacent tissues (Fig. 4a, b, d, e); however, this significant up-regulation is not observed in tumors from patients who never smoked ($n = 10$) or who never smoked or had stopped smoking for at least 15 years ($n = 17$) (Fig. 4a, b, d, e). When − dCt (dCt is Ct of miR-34 subtracted by Ct of housekeeping gene RNU6B) data were examined, we observed that it was not that tumors in the first two categories above (ever smoked/smoke recently) expressed more miR-34b/b*/c than tumors from the opposite categories (never smoke/did not smoke recently), but that the normal adjacent tissues in those who ever smoked or smoke recently down-regulated these three miRNAs (Fig. 4a, b, see underlined mean − dCt values). Previous study in mouse lung showed that cigarette smoke led to down-regulation of several miRNAs including *mir-34b* in cigarette-exposed, non-cancerous lung tissue [38]. Another study in human bronchial epithelia samples demonstrated that miR-34c were expressed at a lower level in cells with histological characteristics of hyperplasia, metaplasia, and mild-to-moderate dysplasia when

Table 3 The association of the expression levels of miR-34-family miRNAs in tumor samples and demographic and clinicopathological data

	Mean fold change of miRNA expression in tumor as normalized to expression in matching normal adjacent tissues (+ SD on the positive side, − SD on the negative side)[a]			
	miR-34a	miR-34b	miR-34b*	miR-34c
Gender				
Male (*n* = 33)	1.52 (+2.82, − 0.99)	1.86 (+2.54, − 1.07)	1.89 (+3.21, − 1.19)	1.61 (+1.88, − 0.87)
Female (*n* = 9)	1.65 (+1.43, − 0.77)	2.75 (+3.46, − 1.53)	3.12 (+4.82, − 1.89)	1.84 (+1.98, − 0.95)
p value**	0.8248	0.5099	0.4548	0.8368
Smoking				
Smoker (*n* = 32)	1.63 (+2.90, − 1.04)	2.40 (+6.17, − 1.73)	2.45 (+9.69, − 1.96)	2.14 (+6.90, − 1.63)
Non-smoker (*n* = 10)	1.31 (+1.67, − 0.73)	1.16 (+10.19, − 1.04)	1.30 (+10.08, − 1.15)	0.75 (+6.19, − 0.67)
p value**	0.5385	0.2040	0.3197	0.0907
Smoking				
Never-to-light smoker (0–10 cigarettes/day) (*n* = 25)	1.57 (+2.48, − 0.96)	1.90 (+8.51, − 1.55)	2.02 (+11.47, − 1.72)	1.52 (+8.14, − 1.28)
Moderate–heavy smoker (11 + cigarettes/day) (*n* = 17)	1.51 (+2.64, − 0.96)	2.21 (+6.17, − 1.63)	2.24 (+7.52, − 1.73)	1.86 (+5.93, − 1.42)
p value**	0.8877	0.7607	0.8555	0.7103
Years since quitting				
< 15 years (*n* = 29)	1.63 (+2.72, − 1.02)	2.57 (+6.84, − 1.87)	2.70 (+11.15, − 2.17)	2.17 (+7.47, − 1.68)
≥ 15 years or never smoke (*n* = 13)	1.37 (+2.30, − 0.86)	1.18 (+7.60, − 1.02)	1.21 (+7.07, − 1.04)	0.86 (+5.70, − 0.74)
p value**	0.5924	0.1378	0.1725	0.1102
Site				
Oral cavity (*n* = 21)	1.76 (+2.97, − 1.10)	2.34 (+3.97, − 1.47)	3.04 (+6.98, − 2.12)	1.89 (+2.82, − 1.13)
Larynx and hypopharynx (*n* = 21)	1.36 (+2.20, − 0.84)	1.74 (+11.21, − 1.51)	1.46 (+10.83, − 1.29)	1.44 (+12.49, − 1.29)
p value**	0.3983	0.5500	0.1761	0.6199
Tumor staging				
I and II (*n* = 6)	1.47 (+4.13, − 1.09)	1.95 (+2.95, − 1.17)	3.04 (+11.08, − 2.39)	1.89 (+5.15, − 1.38)
III and IV (*n* = 36)	1.56 (+2.26, − 0.92)	2.03 (+8.61, − 1.64)	1.98 (+9.67, − 1.64)	1.62 (+7.73, − 1.34)
p value**	0.8957	0.9520	0.5834	0.8383

***p* value is calculated from unpaired Student's *t* test between the two groups shown for each demographic or clinicopathological factor

[a] As standard deviation is calculated from the linear scale (± SD), when differential expression and SD is converted to exponential scale to become fold change and SD of fold change, respectively, SD then has 2 unequal values: one for the positive and one for the negative sides (calculated from $2^{\text{mean+SD}}$ and $2^{\text{mean−SD}}$)

compared to normal bronchia epithelia from non-smokers [39]. Our results agreed with these previous studies as we also observed lower levels of expression of miR-34b/b*/c in normal epithelia surrounding the tumors after exposure to cigarette smoke or recent (quit < 15 years) exposure to cigarette smoke. However, further studies will be needed to determine the mechanism of the effect of cigarette smoking on miR-34 miRNAs expression.

Another correlation of miR-34 expression with clinical data is that miR-34b, miR-34b*, and miR-34c were up-regulated in HNSCC of the oral cavity, but not in HNSCC of the larynx or hypopharynx (Fig. 4c, f). This may be due to a trend of association between *TP53* mutation status and tumor sites in which more oral cavity cancer samples have wild-type *TP53*, but more laryngeal and hypopharyngeal cancer samples have mutations in *TP53* (Table 2). Although this trend of association between *TP53* mutation status

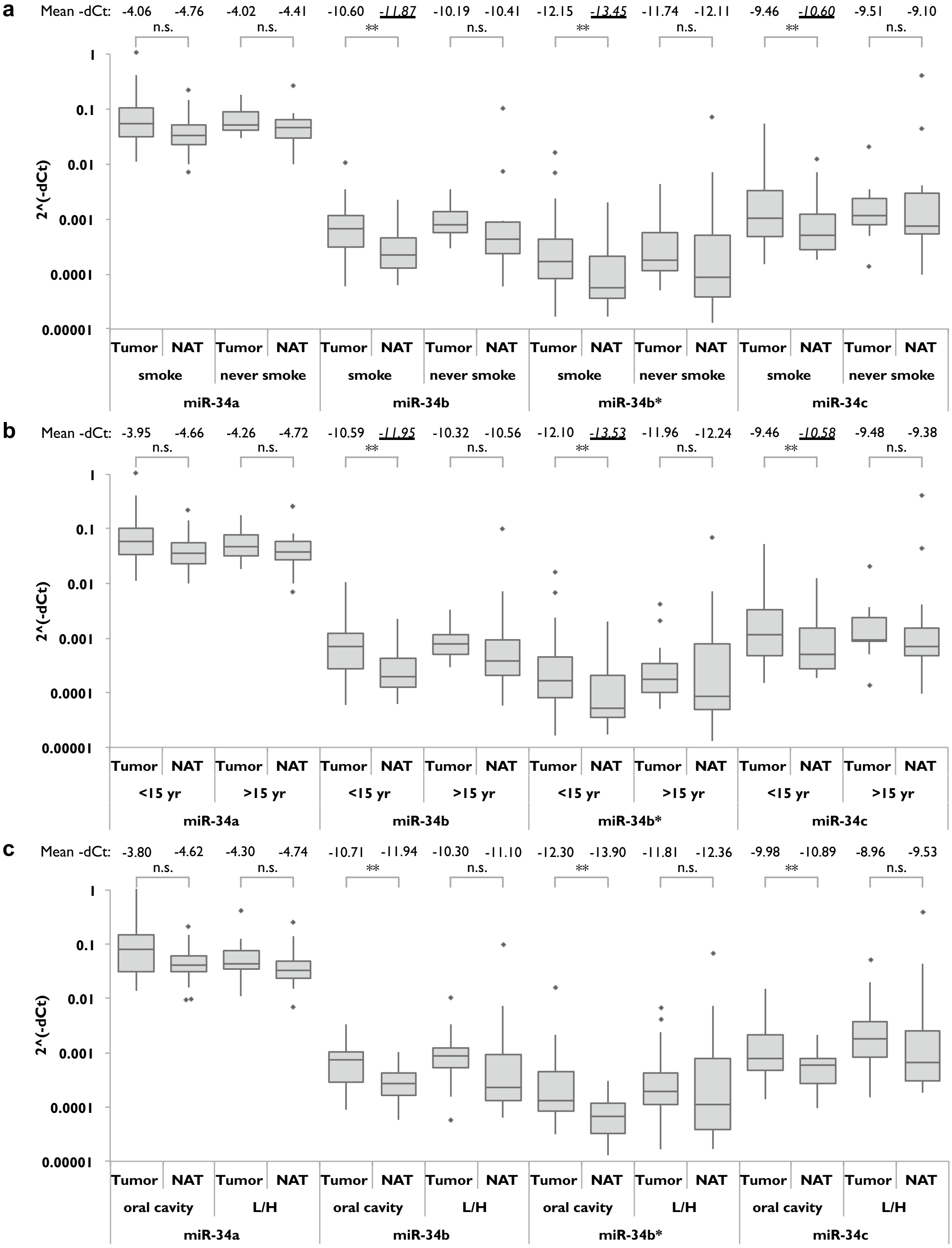

Fig. 4 Expression levels of miR-34b, miR-34b*, and miR-34c in HNSCC samples are correlated with smoking status and tumor sites. Correlation of expression levels of miR-34-family miRNAs with demographic and clinicopathological data is determined by separating patients into two groups based on their history: in **a, d**, the two groups being compared are patients who ever smoked ($n=32$) and those who never smoked ($n=10$); in **b, e**, the two groups are patients who smoked recently (quit smoking less than 15 years ago) ($n=17$) and those who never smoke or did not smoke recently (quit smoking for at least 15 years) ($n=25$); and in **c, f** the two groups are tumors in the oral cavity ($n=21$) and those in the larynx or hypopharynx ($n=21$). **a**–**c** Expression of miR-34-family miRNAs as $2^{-\mathrm{dCt}}$ values, calculated as in Fig. 1. Mean − dCt in each group is shown above its box plot. $^{**}p$ value < 0.01; *n.s.* not statistically significant—as analyzed by paired *t* test. **d**–**f** Expression of miR-34-family miRNAs as fold change, which was calculated as in Fig. 3. Short horizontal lines across dot plots indicate mean fold change for each miRNAs with the values of mean fold change written above. Long dashed lines indicate fold change = 1, which means no change in expression; $^{\#\#}p$ value < 0.01; and *n.s.* not statistically significant—as analyzed by one-sample *t* test when the null hypothesis is no change in expression

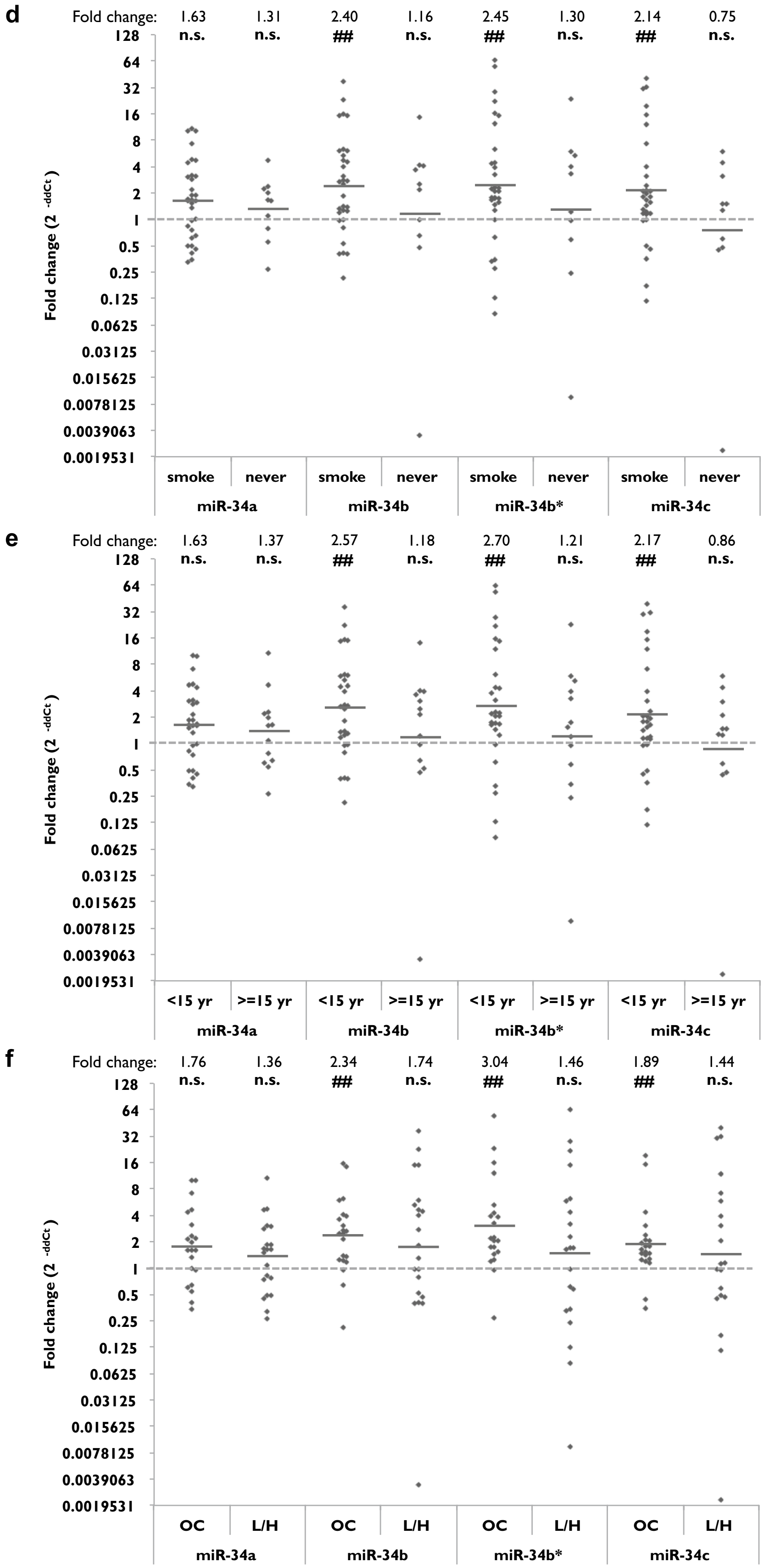

Fig. 4 (continued)

and tumor sites does not reach statistical significance (p value = 0.2146) (Table 2), it may explain in part why there are differences in expression levels of miR-34b, miR-34b*, and miR-34c when comparing samples from different sites of origin. Tumors with WT *TP53* significantly up-regulate miR-34b/b*/c (Fig. 3a, c); similarly, the group of tumors that arose in the oral cavity, in which two-thirds (14 out of 21 samples) have WT *TP53*, significantly expresses more miR-34b/b*/c (Fig. 4c, f). On the other hand, tumors with mutant *TP53* does not significantly up-regulate miR-34b/b*/c (Fig. 3b, d); similarly, the group of tumors that arose in the larynx and hypopharynx, in which ~ 57% (12 out of 21 samples) have mutant *TP53*, does not significantly up-regulate miR-34b/b*/c (Fig. 4c, f).

Conclusion

In conclusion this study examined HNSCC tumor tissue and normal adjacent tissue (NAT) sample pairs from 42 patients and determined their overall genetic characteristics to find that 45% of tumors have *TP53* mutations yet none (0%) is HPV-positive. From clinicopathological data, we find that *TP53* mutations are not associated with gender, age, smoking, tumor sites, and tumor TNM staging. Upon examining miR-34-family microRNAs expression, it is found that HNSCC samples, disregarding their *TP53* mutation status, overexpresses miR-34a, b, and b*. Furthermore, this study shows that expression levels of all four miR-34-family miRNAs are significantly up-regulated in head and neck squamous cell carcinoma with intact *TP53* gene, while those with mutant *TP53* cannot fully up-regulate miR-34-family miRNAs. In addition, while expression of miR-34-family miRNAs is not associated with gender, age, or tumor staging, it is associated with smoking history and tumor sites. miR-34b, b*, and c are up-regulated in tumors from those who ever smoked or smoked recently (quit < 15 years ago) but such up-regulation is not observed in tumors from those who never smoke or did not smoke recently. For tumor sites, miR-34b, b*, and c are up-regulated in tumors of the oral cavity, but such up-regulation is not seen in tumors of the larynx or hypopharynx.

This loss of miR-34 up-regulation only in tumor specimens with mutant *TP53* suggests that their function may be fulfilled by exogenous addition of miR-34. Since miR-34 has been shown to have apoptotic or cell-cycle arrest effect [9–11, 40], it maybe possible to achieve some therapeutic effect by delivering miR-34 mimics to the tumors with mutant *TP53* in order to down-regulate its targets, even without signaling from *TP53* itself [10, 11, 35, 41]. Further experiments are required to test the potential of using miR-34 mimic therapeutics in head and neck squamous cell carcinoma.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Informed consent was obtained from all individual participants included in the study. All procedures performed in this study involving human participants were in accordance with the ethical standards of the Declaration of Helsinki and approved by Siriraj Institutional Review Board (COA no. Si 424/2015).

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