

MiR-21 is a Predictor for Chemoradioresistance and a Novel Therapeutic Target in Head and Neck Squamous Cell Carcinoma

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Abstract

Objective

This study aimed to clarify whether circulating miR-21 represents a predictive biomarker in patients with head and neck squamous cell carcinoma (HNSCC) undergoing chemoradiotherapy, and to investigate the effect of miR-21 inhibitor for chemoradiation in human SCC cells.

Methods

Plasma samples were obtained from 22 patients with HNSCC and 25 non-cancer volunteers. Plasma miR-21 expression was measured using real-time quantitative reverse transcription polymerase chain reaction. The effects of miR-21 inhibitor in human SCC cells were investigated by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry, and Western blot analysis.

Results

Plasma miR-21 expression was higher in HNSCC patients than in control patients ($p < 0.001$). Seven patients with recurrence showed significantly higher plasma miR-21 than the 15 patients without recurrence. Moreover, miR-21 inhibition significantly enhanced cisplatin- or radiation-induced apoptosis. Western blot analysis suggested the programmed cell death 4 (PDCD4) protein as a potential target of miR-21 in relation to apoptosis. Adding miR-21 inhibition to radiation or cisplatin treatment provided clear and potent suppression of tumor cell proliferation.

Conclusion

This study provides new insights into the role of miR-21 as a predictive biomarker for HNSCC treated with chemoradiotherapy, and suggests a potential target to improve the effects of chemoradiotherapy against HNSCC.

Background

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world, with an annual incidence of more than 550,000 cases and around 300,000 deaths each year [1, 2]. Concurrent chemoradiotherapy is the standard treatment for locally advanced (stage III–IV) HNSCC [3], and cisplatin is currently the cytotoxic agent most widely used in combination with radiotherapy for this pathology [3]. According to the Longitudinal Oncology Registry of Head and Neck Carcinoma report for the years 2005 to 2010, 70% of patients with HNSCC in the United States received combination therapy with a cisplatin-based regimen and radiotherapy [4]. Nevertheless, 5-year overall survival (OS) rates for locally advanced HNSCC remain unacceptable, at under 40% [5]. Poor outcomes are associated with chemoradioresistance, leading to local, locoregional or distant failure, and only rarely amenable to further

treatment. Investigation of the molecular mechanisms underlying treatment failure and identification of predictive biomarkers are thus urgently required.

MicroRNAs (miRNAs) are small endogenous, non-coding RNAs of 18–25 nucleotides, acting as post-transcriptional gene expression regulators by destabilizing or inhibiting translation of mRNAs [6]. These miRNAs not only regulate gene-expression, but also are involved in carcinogenesis, such as cell proliferation, apoptosis, angiogenesis [7]. One of the most well-known miRNAs is miR-21, an oncomiR (i.e., an miRNA associated with cancer) for carcinomas such as HNSCC, esophageal squamous cell carcinoma (SCC), gastric carcinoma, lung carcinoma, colorectal carcinoma, breast cancer and pancreatic carcinoma [8, 9, 10, 11, 12]. Increased expression of miR-21 has been deeply involved with poor prognosis of various cancers due to its oncogenic roles in processes such as cell proliferation, migration, and invasion [9]. The potential utility of miR-21 as a predictive biomarker for oncological treatment was recently elucidated, with Atrantes et al. revealing that miR-21 obtained from tissue samples was able to predict response to an organ-preservation protocol based on chemoradiation in HNSCC [13]. However, whether miR-21 circulating in blood can predict chemoradioresistance in patients with HNSCC remains unclear, despite its potential as a strong and useful biomarker in clinical situations.

In addition, emerging evidence has demonstrated that miR-21 modulates the chemosensitivity of cancer cells primarily by targeting phosphatase and tensin homolog (PTEN) or PDCD4 [14, 15, 16, 17]. With regard to the chemoresistance induced by miR-21 in HNSCC, a few reports have shown that miR-21 induced chemoresistance in oral SCC cells [17], but not in pharyngeal or laryngeal cell carcinomas, even though chemoradiotherapy is the standard therapy for advanced oropharyngeal, hypopharyngeal and laryngeal carcinomas. Whether radioresistance is induced by miR-21 in HNSCC remains unclear.

Our aim in the study was to clarify whether circulating miR-21 offers a possible predictive biomarker for patients with locally advanced HNSCC undergoing platinum-based chemoradiotherapy, and to investigate the effects of miR-21 inhibitor together with chemoradiotherapy in human HNSCC cells. Our study revealed circulating miR-21 levels were significantly higher in the group with tumor recurrence after chemoradiotherapy than in the group without recurrence. Next, we showed that miR-21 inhibition significantly suppressed cell proliferation and also promoted cisplatin- and radiation-induced apoptosis in human hypopharyngeal SCC cells. This study provides new insights into the role of plasma miR-21 in HNSCC as a predictive biomarker for chemoradiotherapy, and miR-21 inhibition during chemoradiotherapy may improve the prognosis for advanced HNSCC.

Methods

Patient recruitment and sample collection

A total of 22 patients with advanced HNSCC (21 men, 1 woman; mean age, 60.7 years) and 25 volunteers with no cancer (20 men, 5 women; mean age, 62.3 years) were investigated in this study (Table 1). Control patients had no history of cancers in the preceding 3 years. Peripheral blood samples were

collected from patients with HNSCC who were receiving platinum-based chemoradiotherapy in the Department of Otorhinolaryngology-Head & Neck Surgery at Mie University Hospital, from January 2015 until December 2016. Blood samples were collected before initiating chemoradiotherapy. Within the patient group, we also compared plasma levels of miR-21 between the 15 patients with no recurrence and the 7 patients with recurrence. Clinical characteristics of the 22 patients, including age, sex, stage, tumor site, mean expression level of miR-21 in plasma, and Brinkman index (defined as [number of cigarettes per day] × [number of years during which the patient smoked]), CRP, neutrophil-lymphocyte ratio and SCC antigen are listed in Table 2. This study was approved by the ethics committee at Mie University Graduate School of Medicine (approval nos. 2445 and H2020-232). Written informed consent was obtained from each patient before enrolment in this study.

Cell culture

HNSCC FaDu and SAS cell lines (derived from human hypopharyngeal cell carcinoma and human tongue SCC, respectively) were obtained from RIKEN BRC Cell Bank (Tsukuba, Japan). These HNSCC cell lines were maintained in modified Eagle's medium (MEM) medium or RPMI-1640 medium with 10% fetal bovine serum and antibiotics (1% penicillin, streptomycin), respectively. All human cell lines have been authenticated using short tandem repeat (STR) profiling this year.

RNA extraction from plasma

RNA was extracted from 200 µl of plasma by using an miRNeasy Serum/Plasma Kit (QIAGEN, Hilden, Germany) in the automated QIAcube (QIAGEN) as described previously [10], according to the manufacturer's instructions. A synthetic *Caenorhabditis elegans* miR-39 miRNA mimic (QIAGEN) and carrier RNA (0.94 µg, MS2 bacteriophage total RNA; Roche Applied Sciences, Indianapolis, IN, USA) were spiked-in before RNA extraction. Isolated RNA was eluted in 15 µl of RNase-free water.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) for plasma miR-21

cDNA was synthesized from 5 µl of eluted RNA (containing miRNAs) in HiFlex buffer (QIAGEN), by using an miScript II RT Kit (QIAGEN) as described previously [10]. Next, quantitative RT-PCR was performed using the miScript SYBR Green PCR kit (QIAGEN) and miScript Primer Assays. MiRNA were normalized against the average of four reference miRNAs (miR-423-5p, miR-103a-3p, miR-191-5p, and miR-93), which were used as internal controls in plasma samples. In addition, syn-cel-miR-39 was used to confirm the extraction efficacy of RNA. All amplifications were carried out in an ABI Step One Plus Real-time PCR System (Applied Biosystems, Singapore, Singapore). Amplification curves were analyzed using SDS software version 2.2.2 (Applied Biosystems). Expression levels of mRNA were determined by using the $2^{-\Delta\Delta C}$ method.

Transfection experiments using miR-21 inhibitor

To transiently inhibit miR-21 expression, hsa-miR-21 mirVana™ miRNA inhibitor (Applied Biosystems) or hsa-miR-21 mirVana® mimics (Applied Biosystems) were used to transfect HNSCC cells, as described

previously [18]. Verification of transfection efficiency was conducted using the mirVana™ miRNA Mimic Negative Control (Applied Biosystems) and mirVana™ miRNA Inhibitor Negative Control (Applied Biosystems), respectively. Forward transfections were conducted by mixing siRNA oligonucleotides (70 nM) with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) and Opti-MEM I (Invitrogen) and applying the mixture to cells at 24 h after plating. A series of in vitro assays was conducted after 48–72 h of incubation.

Cell viability assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to assess the anti-proliferative effects of miR-21 inhibitor and proliferative effects of the miR-21 mimic on HNSCC cells, as previously described [19]. Cells were plated at a density of $4 \cdot 10^3$ cells/well into a 96-well plate, transfected with miR-21 mimic or inhibitor, and treated with either 48 h administration of cisplatin at various concentrations (0, 0.2, 1, or 5 $\mu\text{g}/\text{ml}$) or three exposures to radiation at various dose (0, 2, or 4 Gy), and incubated for a total of 72 h. Next, 10 μl of 5-mg/ml MTT (Sigma-Aldrich, St Louis, MO, USA) in 100 μl of medium was added to each well, followed by incubation for 4 h at 37°C. The medium was removed and replaced with 100 μl DMSO, and absorbance values were measured at 570 nm on a Bio-Rad model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Cell apoptosis assay

Quantitative analysis of apoptotic and dead cells was performed by using An Annexin V and Dead Cell Assay Kit (Millipore, Burlington, MA, USA) with a flow cytometer (Muse™ Cell Analyzer; Millipore) as described previously [20], according to the instructions from the manufacturer. After incubating with cisplatin (0, 4, or 6 $\mu\text{g}/\text{ml}$) for 48 h or exposure to radiation (0, 4, or 12 Gy), all cells were harvested and diluted to a concentration of $5 \cdot 10^5$ cells/mL in MEM medium with 2% Fetal bovine serum (FBS). One hundred microliters of Annexin V and Dead Reagent and 100 μl of single cell suspension were then mixed in a microtube and analyzed by using the Muse™ Cell Analyzer (Millipore) after 30 min incubation in the dark at room temperature. All experiments were performed in quadruplicate.

Western blot analysis

After transfection with miR-21 mimic or inhibitor for 48 h, cells were harvested and lysed using RIPA buffer (Cell Signaling Technology, Dancers, MA, USA) supplemented with phenylmethylsulphonyl fluoride (Nacalai Tesque, Kyoto, Japan). Equal amounts of protein were run by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (0.45 μm ; Millipore). Membranes were washed with Tris-buffered saline (TBST) containing 0.1% Tween-20 (Nacalai Tesque) and 5% bovine serum albumin (Sigma-Aldrich, Saint Louis, MO, USA), and incubated overnight at 4°C with primary antibodies (PDCD4 antibody from Cell Signaling Technology, Danvers, MA, USA; β -actin antibody from MP Biomedicals, LLC, Irvine, CA, USA). After washing with TBST, membranes were further incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000; Santa Cruz Biotechnology, Dallas TX, USA) for 1 h at room temperature, and developed by using an electrochemiluminescence system in the end (GE Healthcare, Little Chalfont, UK). Protein bands were

detected by an LAS4000mini imaging system (Fujifilm, Tokyo, Japan), and band intensities of Western blots were quantitatively measured by calculating integrated grayscale densities in consistently sized windows incorporating each band using ImageJ version 1.48 software as described previously [21].

Statistical analysis

All statistical analyses were performed with SPSS version 23 statistical software. Two-group comparisons of ΔCt values between plasma samples from controls and HNSCC patients, as well as relapse-free and recurrent patients, were performed by using the Mann-Whitney U -test. Receiver operating characteristic (ROC) curves were chosen to evaluate the predictive power of miR-21 for tumor recurrence and to obtain the miR-21 cut-off values offering maximal sensitivity and specificity. The Kaplan-Meier method with log-rank comparison was selected to calculate OS, measured from the date chemoradiotherapy was initiated until the date of death from any cause (i.e., cancer-unrelated deaths were not censored), or last known follow-up for patients who were still alive. Statistical differences between groups were calculated by χ^2 test, unpaired t-test, Mann-Whitney test, and one-way ANOVA with post-hoc multiple comparisons. Values of $p < 0.05$ were considered to be a statistical significance.

Results

Plasma miR-21 level and OS rate in HNSCC patients treated with chemoradiotherapy

First, peripheral blood samples were collected from 22 patients with advanced HNSCC treated using platinum-based chemoradiotherapy and 25 healthy subjects without HNSCC, and levels of plasma miR-21 expression were detected by qPCR. MiR-21 is an abundantly expressed miRNA in mammalian cells. MiR-21 showed low expression level in steady state, and it would be upregulated in malignancy [22].

As expected, relative levels of miR-21 were significantly higher in HNSCC patients than in control subjects (Fig. 1A; $p < 0.001$). Next, we sought to determine whether expression levels of plasma miR-21 were associated with tumor recurrence after chemoradiation. Our results demonstrated a significant difference in plasma miR-21 levels between the tumor-free and recurrent groups (Fig. 1B; $p < 0.05$). Factors including age, sex, stage, subsite, Brinkman index, CRP, neutrophil-lymphocyte ratio and SCC antigen were compared between the tumor-free and recurrent groups. The difference between groups was not significant ($p > 0.05$; **Table 2**). Though we also examined the correlation plasma miR-21 and several clinical parameters like CRP, neutrophil-lymphocyte ratio and SCC antigen, there were no correlation. This result implied that plasma miR-21 could offer a predictive biomarker for tumor recurrence after chemoradiotherapy in HNSCC. We generated the ROC curve to assess the potential utility of plasma miR-21 as a biomarker for early detection of recurrent HNSCC after chemoradiotherapy (Fig. 1C). The area under the ROC curve (AUC) for plasma miR-21 was 0.7771. Using a cut off value of 1.295, miR-21 showed 85.7% sensitivity and 66.7% specificity. These findings highlighted the potential for plasma miR-21 as a useful biomarker for predicting recurrent HNSCC after chemoradiotherapy.

Several reports have suggested miR-21 as a candidate prognostic biomarker for HNSCC [12, 13, 23], and a relationship between high miR-21 expression and tumor recurrence was identified in the present study. However, whether tumor recurrence exerts a big impact on prognosis has remained unclear. We therefore assessed the correlation between tumor recurrence and OS. The results demonstrated a significant difference in OS between the tumor-free and recurrent groups (Fig. 1D), in turn suggesting the importance of miR-21 as a prognostic biomarker.

Increased sensitivity to radiotherapy in HNSCC cells by miR-21 inhibitor

Having identified plasma miR-21 expression level as increased in the group with recurrent HNSCC comparing to the tumor-free group in this study, the role of miR-21 in chemoradiotherapy for HNSCC is still unknown. We hypothesized that miR-21 would play an important role in chemoradioresistance to platinum-based chemoradiotherapy for HNSCC. First, we confirmed expression levels of miR-21 by RT-qPCR analysis in human HNSCC cells (FaDu cells derived from hypopharyngeal SCC) with transfection of either miR-21 mimic or miR-21 inhibitor. As expected, we could confirm that miR-21 inhibitor suppressed miR-21 expression (Fig. 2A) and miR-21 mimic induced miR-21 expression (Fig. 3A). Although miR-21 has been shown to be involved in the radioresistance of several types of cancer, including breast cancer, lung cancer, cervical cancer, and glioma [24, 25, 26, 27], whether miR-21 inhibitor enhances the radiosensitivity of human HNSCC cells is still unclear. We therefore next assessed the effects of miR-21 inhibitor on treatment with radiation by performing an MTT proliferation assay. We determined the RT dose for MTT assay because patients with HNSCC are treated with single dose 2Gy (totally 60–70 Gy in 30–35 fractions over 6–7 weeks) in conventional radiotherapy [28]. Cell proliferation was significantly suppressed by miR-21 inhibitor compared with radiation alone (Fig. 2B, 2C). Similar results were also observed in the SAS cell line, as human tongue SCC cells (Fig. 2F). Consistent with these results, MTT assay results showed radioresistance with miR-21 mimic transfection (Fig. 3B). Taken together, these results suggest that miR-21 is deeply involved in the radiosensitivity of HNSCC cells.

Increased sensitivity to chemotherapy in HNSCC cells by miR-21 inhibitor

With regard to the chemoresistance of HNSCC by miR-21, we also investigated whether miR-21 is involved in the chemosensitivity of HNSCC treated with miR-21 inhibitor or mimic using the MTT assay. According to Nagai et al [29], high dose CDDP administration (80mg) after continuous infusion over 4 hours and over 2 hours provided $1.39 \pm 0.79 \mu\text{g/ml}$ and $2.22 \pm 0.90 \mu\text{g/ml}$ of plasma concentration of unchanged CDDP, respectively. For this reason, we selected CDDP concentration at 0.2, 1, 5 $\mu\text{g/ml}$ for MTT assay. Cell proliferation was significantly decreased by cisplatin treatment in a concentration-dependent manner, and miR-21 inhibitor enhanced the effects of cisplatin treatment in a similar manner to that with radiation (Fig. 2D, 2E). Similar results were also observed in SAS cells (Fig. 2G). Supporting these findings, miR-21 mimic transfection induced obvious chemoresistance in HNSCC (Fig. 3C). Our results thus clearly demonstrated that miR-21 is also involved in chemoresistance for HNSCC cells.

Apoptotic effects of miR-21 inhibitor in HNSCC cells

We provided direct evidence that miR-21 plays an important role in HNSCC chemoradioresistance. However, the mechanisms underlying the distinct roles of miR-21 inhibitor in regulating chemoradioresistance of HNSCC remain elusive. We further analyzed the effects of miR-21 inhibitor on apoptosis from treatment with either cisplatin or radiation by flow cytometry after annexin V and 7-amino-actinomycin D staining. The results demonstrated that FaDu cells transfected with miR-21 inhibitor showed synergistic enhancement of apoptosis compared with either cisplatin alone or radiation alone (Fig. 4A-4D). We also used flow cytometry to evaluate the effects of miR-21 mimic, revealing significant suppression of the apoptosis induced by either radiation or cisplatin treatment (Fig. 4E-4H).

PDCD4 as a possible target of miR-21

PDCD4 has been reported as a target molecule related to drug sensitivity in various cancers [30,31], so we investigated protein levels of PDCD4 following transfection of miR-21 inhibitor or mimic in FaDu cells by Western blot analysis. As expected, we could confirm that miR-21 inhibitor upregulated PDCD expression, while miR-21 mimic decreased PDCD4 expression (Fig. 5A-5D). This result demonstrated that PDCD4 was definitely regulated by miR-21 expression.

Discussion

To improve the prognosis of HNSCC, finding useful biomarkers to estimate chemoradiosensitivity in the individual HNSCC patient is crucial. A major finding in this study was the direct evidence that expression levels of plasma miR-21, not from tissue samples in HNSCC patients with recurrence were significantly higher than those in patients without recurrence. As expected, the recurrent HNSCC group showed poorer OS (Fig. 1C). Our results clearly identified plasma miR-21 at the time-point of pretreatment as a potentially useful predictor of HNSCC susceptibility to chemoradiotherapy. Several studies have described circulating miRNAs as predictors of the efficacy of chemoradiotherapy in various cancers, including rectal cancer [32,33], cervical SCC [34], nasopharyngeal carcinoma [35], HNSCC [36], and esophageal carcinoma [37]. In addition, in the field of HNSCC as prognostic biomarkers for chemoradiotherapy, circulating miR-1290 [36] has been reported as useful for oral SCC, and circulating miR-744 as useful for nasopharyngeal carcinoma [38]. However, no reports have identified circulating miR-21 as predictive of chemoradiosensitivity in HNSCC patients. Our study may thus bring new insights into the novel roles of plasma miR-21 in predicting chemoradioresistance for HNSCC patients.

In addition, further interesting evidence was provided for the involvement of miR-21 in suppressing chemoradiosensitivity in HNSCC in this study. Even though miR-21 is well known as an oncomiR for various types of carcinoma [10, 11, 12], whether elevated miR-21 levels are associated with chemoradioresistance and the influence of miR-21 on radioresistance in HNSCC have remained unclear. The present study provides first-hand evidence that miR-21 inhibitor enhanced the anti-proliferative effects of radiation in HNSCCs, including FaDu cells and SAS cells, which means hypopharyngeal squamous cell carcinoma and tongue squamous cell carcinoma cell lines, except nasopharyngeal

carcinoma cell line. [39]. In addition, miR-21 inhibitor was also found to enhance the apoptotic effects of radiation, but had little effect by miR-21 inhibitor alone. These data confirmed that miR-21 caused radioresistance by increasing cell proliferation and decreasing apoptosis, identifying a potential therapeutic target for reducing radioresistance. Additional experimental significance of our findings was that miR-21 inhibitor also enhanced the anti-proliferative effects of cisplatin in HNSCCs, including FaDu and SAS cell lines. A few reports have described miR-21 as modulating chemosensitivity to cisplatin for oral SCC [17, 40], but no report about oropharyngeal or hypopharyngeal cell carcinoma. Because chemoradiotherapy is the standard therapy for advanced oropharyngeal or hypopharyngeal cell carcinomas, but not oral SCC, the present study offers useful information for clinical strategies. Further, miR-21 inhibitor also enhanced the apoptotic effects of radiation, but had little effect when administered alone. Collectively, these data demonstrated that miR-21 was deeply involved in the chemoradioresistance of HNSCC, suggesting miR-21 as a potential target to maximize the effects of chemoradiotherapy on HNSCC.

Finally, our data in this study implicated the involvement of PDCD4 as a target of miR-21 (Fig. 5A-5D). Knockdown of miR-21 clearly elevated PDCD4 expression on Western blotting. Logically, decreases in PDCD4 by miR-21 would suppress tumor cell apoptosis, contributing to chemoradioresistance [41, 42, 43]. There has been shown that PDCD4 exerts its activity by interacting with eIF4A and eLF4G to suppress mRNA translation and further inhibit the growth and proliferation of tumors [30]. It is one of the possible mechanism to inhibit cell proliferation by miR-21 inhibitor in this study. Unexpectedly, miR-21 did not influence PTEN in FaDu cells in this study[44, 45] (data not shown). In addition to PDCD4, a number of targets of miR-21 have been already reported including phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which is a tumor suppressor gene encoding a phosphatase that regulates cell cycle Akt and p53 activity, and reversion-induced cysteine-rich protein (RECK) and tissue inhibitor of metalloproteinases 3 (TIMP3), which are suppressors of malignancy and inhibitors of matrix metalloproteinases. [22] Our studies are insufficient to provide a full understanding of the mechanisms by which miR-21 induces chemoradioresistance in HNSCC. This question needs to be addressed in future investigations.

Conclusion

In conclusion, the present study appears to emphasize the importance of circulating miR-21 as a predictive biomarker in chemoradiotherapy for HNSCC. Our data reveal the importance of miR-21 to chemoradioresistance in HNSCC, and provide new insights into novel roles of miR-21 for diagnosing and treating HNSCC.

Abbreviations

AUC: area under the curve

CI: confidence interval

CT: computed tomography

CRT: chemoradiotherapy

CRP: C-reactive protein

HNSCC: head and neck squamous cell carcinoma

miRNA: microRNA

miR-21: microRNA 21

mRNA: messenger RNA

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PDCD4: programmed cell death 4

PTEN: phosphatase and tensin homolog deleted from chromosome 10

ROC: receiver operating characteristic

RT: radiotherapy

qRT–qPCR: quantitative reverse transcription polymerase chain reaction

SE: standard error

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee at Mie University Graduate School of Medicine (approval nos. 2445 and H2020-232). Written informed consent was obtained from each patient before enrolment in this study.

Consent for publication

All data described in this manuscript are original. None of the material has been published or is being considered for publication elsewhere.

Conflict of interest

The authors have no conflicts of interest to declare.

Availability of data and material

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

HI, YO, YT and MM designed the experiments. BH, FH and SAS collected samples and measured plasma miR-21. HI, CY and YO performed the research. HI and KT wrote the manuscript.

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Tables

Table 1: Comparison between control subjects (n=25) and advanced HNSCC (n=22)

	Control subjects	advanced HNSCC
Patient' s number	25	22
Age (average)	62.3	60.7
Sex		
male	20	21
female	5	1
serum miR-21 (average)	0.95	1.41
tumor origin		
hypopharynx		10
oropharynx		8
epipharynx		2
maxillary sinus		2
Stage		
III		8
IV	14	
regimen		
CDDP	1	
CDDP +5 - FU	17	
CBDCA+5-FU	4	

Table 2: Comparison between relapse free cases (n=15) and recurrent cases (n=7)

		relapse free cases n=15	recurrent cases n=7	p value
mean age		61.7y. o.	58.7y. o.	0.832
sex	male	14	7	0.259
	female	1	0	
plasma miR21	(mean)	1.27	1.70	<u>0.04</u>
stage	III	6	2	0.49
	IV	9	5	
subsite	oro- or hypopharynx	13	5	0.378
	others	2	2	
brinkman index	≤ 400	4	1	0.651
	> 400	11	5	
CRP		0.413	2.059	0.49
neutrophil-lymphocyte ratio		2.75	2.49	0.49
SCC antigen		1.66	2.33	0.112

Figures

Fig.1A

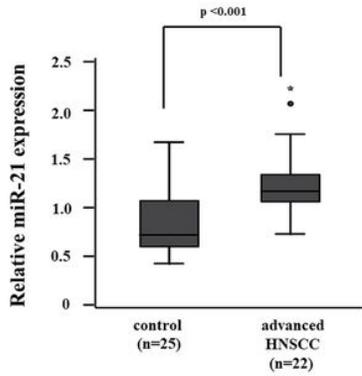


Fig.1B

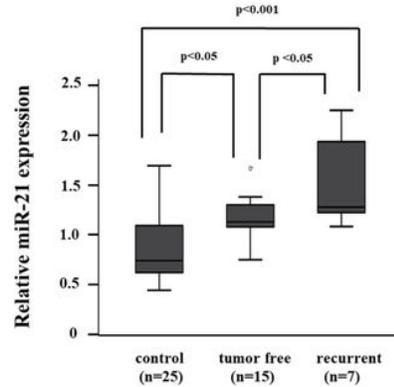


Fig.1C

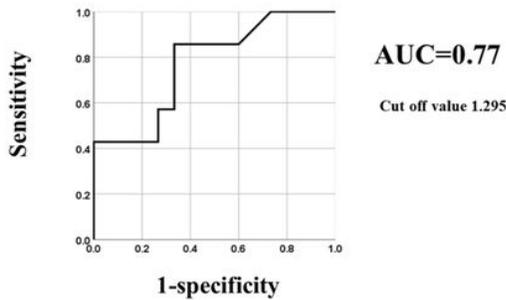


Fig.1D

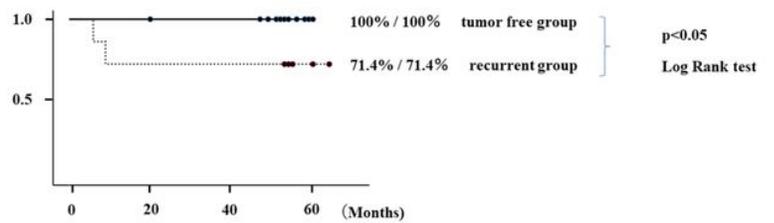


Figure 1

Plasma miR-21 levels in HNSCC patients A) Levels of miR-21 in plasma samples from 25 control subjects and 22 HNSCC patients are measured using RT-qPCR methods. The y-axis represents the relative miR-21 level. Expression level of miR-21 is calibrated to obtain fold change. Statistical significance is given for the comparison between HNSCC patients and control individuals. B) Levels of miR-21 in plasma samples from 25 control subjects, patients who are tumor-free after chemoradiotherapy (n=15) and recurrent patients (n=7) as measured by RT-qPCR methods. Significant differences are determined using one-way ANOVA with post-hoc correction. C) ROC curve analysis. Plasma miR-21 yields an AUC of 0.771, with 85.7% sensitivity and 66.7% specificity in distinguishing recurrent HNSCC patients from tumor-free patients after chemoradiation at a cutoff value of 1.295. D) OS according to HNSCC recurrence by Kaplan-Meier methods. Mean follow-up period is 46.4 months. A significant difference is seen between groups by log-rank testing.

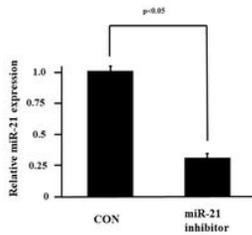
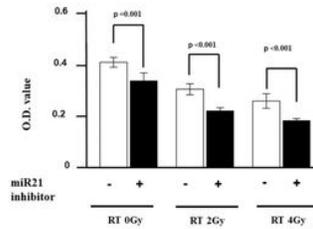
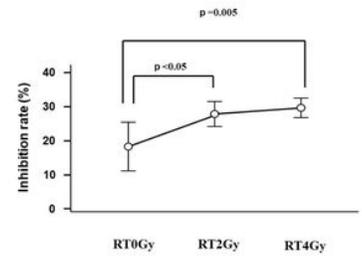
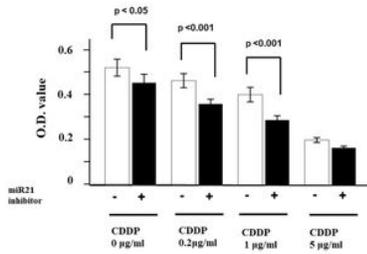
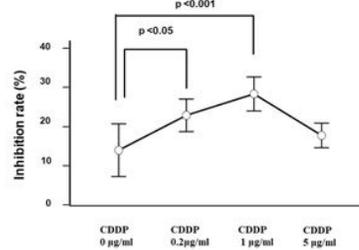
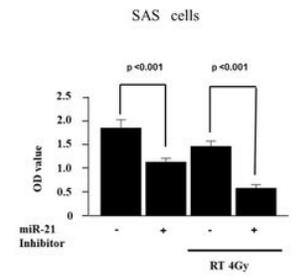
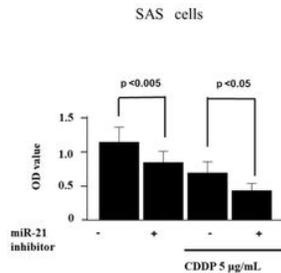
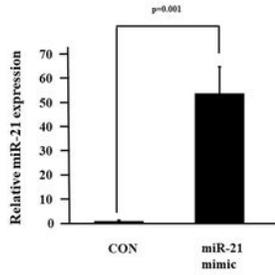
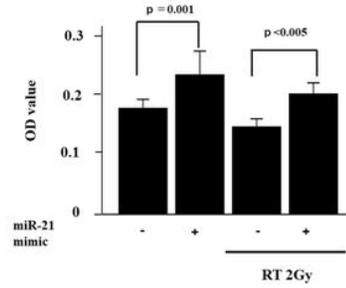
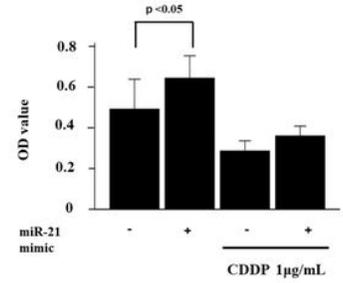
Fig.2A**Fig.2B****Fig.2C****Fig.2D****Fig.2E****Fig.2F****Fig.2G**

Figure 2

Effects of miR-21 inhibitor on radiosensitivity and chemosensitivity A) Knockdown of miR-21 by miR-21 inhibitor in FaDu cells is measured using RT-qPCR methods. Approximately 70% knockdown of miR-21 expression is obtained by 48 h after transfection of 70 nM of miR-21 inhibitor (n=3). B) Anti-proliferative effect of radiation with miR-21 inhibitor on MTT assay. FaDu cells transfected with miR-21 inhibitor are treated with various dose of radiation (0, 2 or 4 Gy) three times (n=6). C) Inhibition rate for radiation with miR-21 inhibitor is assessed by MTT assay. Inhibition rate is enhanced by 2-Gy radiation with miR-21 inhibitor. D) Anti-proliferative effect of cisplatin with miR-21 inhibitor on MTT assay. FaDu cells transfected miR-21 inhibitor are treated with various concentration of cisplatin (0, 0.2, 1 or 5 µg/ml) for 48 h (n=6). E) Inhibition rate for cisplatin with miR-21 inhibitor is assessed by MTT assay. Inhibition rate is enhanced by 1 µg/ml of cisplatin with miR-21 inhibitor. F, G) Effects of miR-21 inhibitor are also confirmed in SAS cells, as a human tongue squamous cell carcinoma line. Anti-proliferative effects from co-treatment with radiation and miR-21 inhibitor are demonstrated in the left panel. Effects from co-treatment with cisplatin and miR-21 inhibitor are shown in the right panel. Values are presented as mean ± standard error. Data are representative of three independent experiments.

Fig.3A**Fig.3B****Fig.3C****Figure 3**

Effects of miR-21 mimic for radiosensitivity or chemosensitivity: A) Overexpression of miR-21 on FaDu cells is measured using quantitative PCR methods. Massive induction of miR-21 expression is seen 48 h after transfection with 70 nM of miR-21 mimic (n=3). B) MiR-21 mimic shows radioresistance on MTT assay (n=6). Anti-proliferative effects of radiation (2 Gy, three times) are counteracted by miR-21 mimic 72 h after transfection. C) MiR-21 mimic shows chemoresistance on MTT assay (n=6). Anti-proliferative effect of cisplatin (1 µg/ml for 48 h treatment) is counteracted by miR-21 mimic at 72 h after transfection. Values are presented as mean ± standard error. Data are representative of three independent experiments.

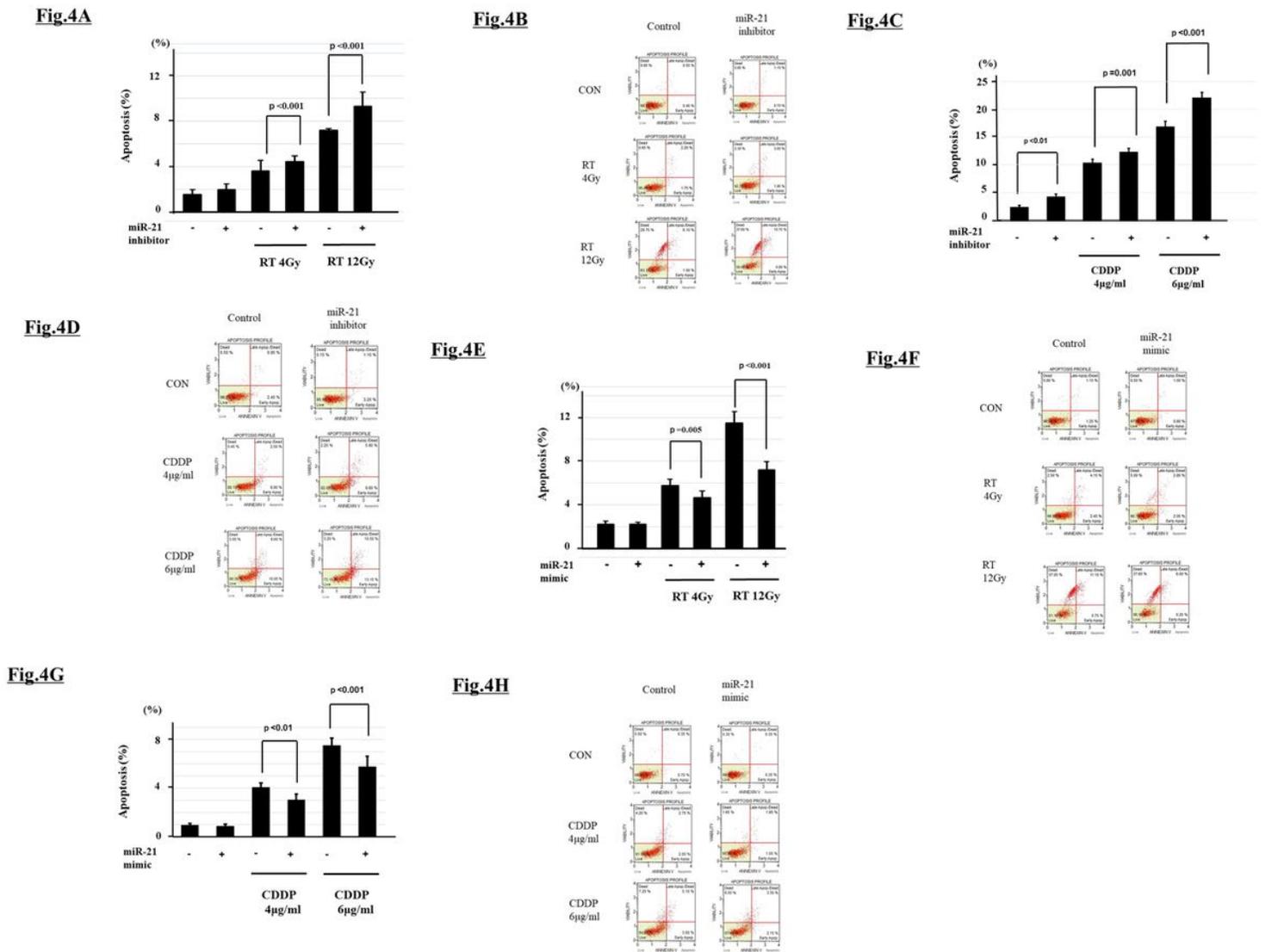


Figure 4

Apoptotic effects of miR-21 inhibitor in HNSCC A) Apoptosis assay performed to measure the population of apoptotic FaDu cells after exposure to radiation with miR-21 inhibitor. Apoptosis rates are measured by annexin V and 7-amino-actinomycin D (7-AAD) staining, and the proportion of apoptotic cells is calculated. Rate of apoptotic cells is significantly enhanced by miR-21 knockdown with radiation (n=6 per group) as shown in the left panel. B) Representative graphs of flow cytometry for miR-21 knockdown with radiation. Upper left: no treatment; middle left: 4-Gy radiation alone; lower left: 12-Gy radiation alone; upper right: miR-21 inhibitor alone; middle right: miR-21 inhibitor + 4-Gy radiation; lower right: miR-21 inhibitor + 12-Gy radiation. C) Apoptotic effects of cisplatin are enhanced by co-treatment with miR-21 inhibitor. Rate of apoptotic cells is significantly enhanced by miR-21 knockdown with cisplatin (n=6 per group). D) Representative graphs of flow cytometry for miR-21 knockdown with cisplatin. E) Apoptotic effects of radiation are significantly suppressed by overexpression of miR-21. Rate of apoptotic cells is significantly decreased by miR-21 overexpression compared to RT alone (n=6 per group). F) Representative graphs of flow cytometry for miR-21 overexpression with radiation. G) Apoptotic effects of

cisplatin are significantly inhibited by miR-21 overexpression. Rate of apoptotic cells is significantly inhibited by miR-21 overexpression compared to cisplatin alone (n=6 per group). H) Representative graphs of flow cytometry for miR-21 overexpression with cisplatin. Values are presented as mean \pm standard error. Data are representative of three independent experiments.

Fig.5A

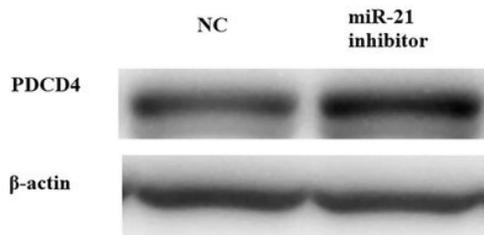


Fig.5B

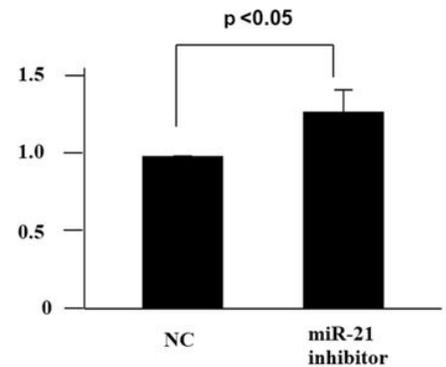


Fig.5C

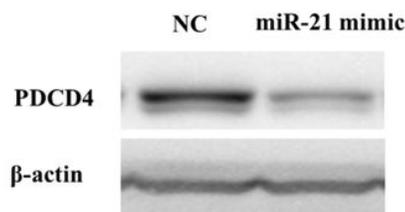


Fig.5D

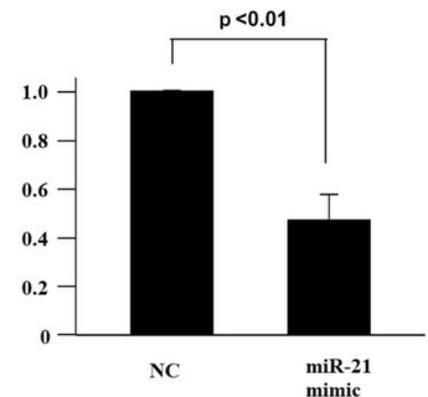


Figure 5

Effects of miR-21 on expression of PDCD4 A) Administration of miR-21 inhibitor increases expression of PDCD4 in FaDu cells, as assessed by Western blot analysis. Full length Western blot data of Fig5A was provided as supplementary data 1 (Supple 1). B) PDCD4 protein expression level after transfection of miR-21 inhibitor is standardized against the level of GAPDH and presented as the relative intensity. C) Overexpression of miR-21 suppresses expression of PDCD4 in FaDu cells, as assessed by Western blot analysis. Full length Western blot data of Fig5C was provided as supplementary data 2 (Supple 2). D) PDCD4 protein expression level with overexpression of miR-21 is standardized against the level of GAPDH and presented as the relative intensity. Values are presented as mean \pm standard error. Data are representative of three independent experiments.

Supplementary Files

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