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Research Article

A comparative study of single or dual treatment of theranostic ^{188}Re-Liposome on microRNA expressive profiles of orthotopic human head and neck tumor model

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Keywords: ^{188}Re-liposome; HNSCC; Cerenkov luminescent imaging; microRNA expressive profile; prognostic factors

Abstract

Background: ^{188}Re-liposome has been used for evaluating the theranostic efficacy on human head and neck squamous cell carcinoma (HNSCC) at preclinical stages. Here we furthercompared the microRNA expressive profile in orthtopic HNSCC tumor model exposed to ^{188}Re-liposome.

Methods: A single dose or dual doses of ^{188}Re-liposome was intravenously injected into tumor-bearing mice followed by the Cerenkov luminescent imaging (CLI) for monitoring the accumulation of ^{188}Re-liposome in tumors. The microRNA expressive profile was generated using the TaqMan® OpenArray® Human MicroRNA Panel followed by the DIANA mirPath analysis, KEGG signaling pathways prediction, and Kaplan-Meier survival analysis for predicting the prognostic role of ^{188}Re-liposome affected microRNAs.

Results: Dual doses of ^{188}Re-liposome exhibited a better tumor suppression than a single dose of ^{188}Re-liposome, including reduced tumor size, Ki-67 proliferative marker, and epithelial-mesenchymal transition (EMT) related factors. The microRNA expressive profiles showed that 22 microRNAs and 19 microRNAs were up-regulated and down-regulated by dual doses of ^{188}Re-liposome, respectively. Concomitantly, these two groups of microRNAs were inversely regulated by a single dose of ^{188}Re-liposome accordingly. These microRNAs influenced most downstream genes involved in cancer related signaling pathways. Further, miR-520e and miR-522-3p were down-regulated whereas miR-186-5p and miR-543 were up-regulated by dual doses of ^{188}Re-liposome, and they separately affected most of genes involved in their corresponding pathways with high significance. Additionally, high expressions of miR-520e and miR-522-3p were associated with lower survival rate of HNSCC patients.

Conclusion: MicroRNA expression could be used to evaluate the therapeutic efficacy and regarded prognostic factors using different doses of ^{188}Re-liposome.

Introduction

Radiogenomics is a rising field of modern applications for radiomics linking to the genetic profile of tumor progression to better predict the therapeutic responses from diagnosis to prognosis. A recent review has summarized the applications of radiogenomics in a variety of human cancers [1]. It also directly influences the gene expressive profile of target tissues because of the radiotherapy induced cytotoxicity [2]. Radiopharmaceutical is an ideal candidate for monitoring

the efficacy of drug targeting and therapy using preclinical or clinical imaging modalities. However, radiopharmaceutical is little applied in radiogenomics.

Theranostics is defined as specific targeted diagnosis and therapy using a material with both effects on tumor treatment. Nuclear medicine plays an important role in theranostics as several types of radiopharmaceuticals emit both γ-rays and high energy β particles for diagnosis and therapy, respectively [3]. For instance, rhenium-188 (^{188}Re) emits 85% of 2.12MeV β particles and 15% of 155keV γ-rays during decay, so it belongs to a theranostic radionuclide as well [4]. It is also an attractive and affordable radiopharmaceutical because of its short half-life and on-demand availability using a tungsten-188/rhenium-188 generator [4]. Moreover, the atomic radius of ^{188}Re is similar to technetium that has been widely used in clinics [5,6]. The tissue penetration of emitted β particles is about 10 mm, suggesting that it is suitable for the treatment of large-sized or mid-late stage tumors [7]. Accumulated literature have demonstrated that liposome embedded ^{188}Re (^{188}Re-liposome) is able to target human colorectal cancer, glioblastomas, esophageal cancer, head and neck cancer and lung cancer using the xenograft tumor model [8-12]. Tumor accumulation of ^{188}Re-liposome is passive on the behalf of the enhanced permeability and retention (EPR) effect, which is dependent on the mal-formation of blood vessels surrounding tumors [13,14]. Previous studies have demonstrated that ^{188}Re-liposome could influence the gene expression in human head and neck squamous cell carcinoma (HNSCC), and the *let-7* family of microRNA mediated gene expressive profile was significantly involved in this treatment [12]. Therefore, this finding intrigues us to investigate whether ^{188}Re-liposome would also influence the microRNA expressive profile.

Although ^{188}Re-liposome exhibited tumor accumulative property in HNSCC, the therapeutic efficacy was moderate. Specifically, regrowth of xenograft tumors were detected when they were treated by a single dose of ^{188}Re-liposome but not by repeated doses [15]. As chemoresistance is known to be associated with a series of molecular regulation [16], we are interested in investigating the gene expressive profiles of HNSCC tumors treated with ^{188}Re-liposome. In this study, we exploited the open arrays of microRNA to analyze over 700 microRNA in orthotopic HNSCC tumor treated with a single dose or repeated doses of ^{188}Re-liposome. The interested microRNAs were also subjected to survival analysis. The results of ^{188}Re-liposome modulated expression of microRNA were discussed.

Materials and methods

Cell line

Human FaDu head and neck squamous cell carcinoma cells (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI-1640 (Life Technologies Inc., Carlsbad, CA, USA) medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamate, 50 unit/ml penicillin and 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA). The pH of medium was adjusted by sodium bicarbonate. Cells were incubated at 37 °C in a humidified incubator with 5% CO_2, and passaged every two days.

Preparation of Rhenium-^{188}liposomal drug

The procedures of ^{188}Re-liposome preparation of validation were followed as described previously [17]. The dose of each injection is 640 μCi corresponding to 80% maximum tolerated dose (MTD) [9].

HNSCC orthotopic tumor model

Four-week old male BALB/c nude mice were used to establish the orthotopic tumor model (National Laboratory Animal Center of Taiwan, Tainan, Taiwan). FaDu cells (1 x 10^6) were resuspended in 100 μl of OPTI-MEM and injected into the buccal positions of mice (N = 5) using a 27G insulin needle. Tumors could form about 3 weeks after tumor implantation. The study has been approved by the Institutional Animal Care and Utilization Committee (IACUC) of National Yang-Ming University (case no. 1061010).

Cerenkov luminescent imaging (CLI) and tumor resection

CLI was performed at 24 hours after the administration of ^{188}Re-liposome. The signals were acquired by the *in vivo* Imaging System (IVIS 50, Perkin Elmer Inc., Waltham, MA, USA). Regions of interest (ROIs) were delineated on the tumor around the mouth. For evaluation of tumor response to ^{188}Re-liposome, resection of tumors with various treatment were performed and compared by size.

Immunohistochemical (IHC) staining

The procedures of IHC was followed by our previous report [15]. Fixed tissue sections were incubated with anti-Ki-67 antibody (MAB4190, EMD Millipore, Billerica, MA, USA) at 4 °C overnight. The slides were rinsed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (EnVisionTM+Dual Link System-HRP(DAB+), K4065, Dako), and developed in 3',3'-diaminobenzidine (DAB+) substrate chromogen and counterstained with Mayer's hematoxylin (ScyTek Laboratories, Utah, USA). The Ki-67 positivity index was quantified after the digitalization of the slides and calculated by the online ImmunoRatio automated counting program (http://153.1.200.58:8080/immunoratio/) [18].

Western blot analysis

Tumors were resected from the tumor-bearing mice after 4 weeks of ^{188}Re-liposome treatment, and lysed using the T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) containing 1% of protease inhibitor cocktail (Sigma-Aldrich). Protein lysates were run on 8% - 12% sodium dodecyl sulfate –polyacrylamide gel electrophoresis (SDS-PAGE). The fractionated proteins

were transferred based on previously [19]. The antibodies included E-cadherin (GTX100443), Slug (GTX128796), ZEB-1(GTX105278), vimentin (GTX100619), Snail (GTX100754) were from GeneTex (Genetex Inc. Irvine, CA, USA). Antibody against GAPDH (MA5-15738) was from Sigma (Sigma-Aldrich Co, St. Louis, MO, USA).

MicroRNA expressive profiling analysis

TaqMan® OpenArray® Human MicroRNA Panel (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to detect the microRNA expression in orthotopic HNSCC tumors treated with different regimens of ^{188}Re-liposome and compared to untreated control. The operation was performed in the Sequencing Core Facility of National Yang Ming University Genome Center (YMGC). For analysis, the expression levels of the two experimental groups (dual doses and single dose of ^{188}Re-liposome) were defined by the Mean Relative Quantification (RQ) normalized to the control group. The expression level changes between the groups were demonstrated by log2 ratio. All the identified assay id of each miRNA probes was converted to miRBase ID (v22) referring to the manufacturer's handbook. The lists were input to DIANA mirPath v.3 (http://snf-515788.vm.okeanos.grnet.gr/) for Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, referencing the DIANA-MicroT prediction [20]. The overall involvement and the co-regulated gene prediction were concluded with p - value threshold of 0.05 and MicroT threshold of 0.8 by genes union and genes intersection function, respectively.

Survival analysis for microRNA

The association of microRNA of interest with public human HNSCC microRNA database was determined by an online Kaplan-Meier plotter [21]. In the database, the miRNA subsystems include 11k samples from 20 different cancer types, which include 523 human HNSCC cases. The significance of microRNA associated survival rate was determined by a log-rank test.

Statistics

Data were represented as the mean ± S.D. from triplicate independent results, and the statistical analysis was performed using t-test. The statistical significance was set at $p < 0.05$.

Results

Effects of single dose and dual doses of ^{188}Re-liposome on the growth of orthotopic HNSCC tumor model

The regimen of ^{188}Re-liposome treatment and the timeline of imaging evaluation and tumor resection on HNSCC tumor-bearing mice were schemed (Figure 1A). Because ^{188}Re-liposome emits high energy β-particles, the drug accumulation at tumor site can be detected by the Cerenkov luminescent imaging (CLI) *in vivo*. Compared to the untreated control, both strategies of ^{188}Re-liposome treatment showed CLI signals in tumor-bearing mice (Figure 1B). Although dual doses did not exhibit increased accumulation of ^{188}Re-liposome at tumor lesion, the tumor size was apparently smaller than that treated

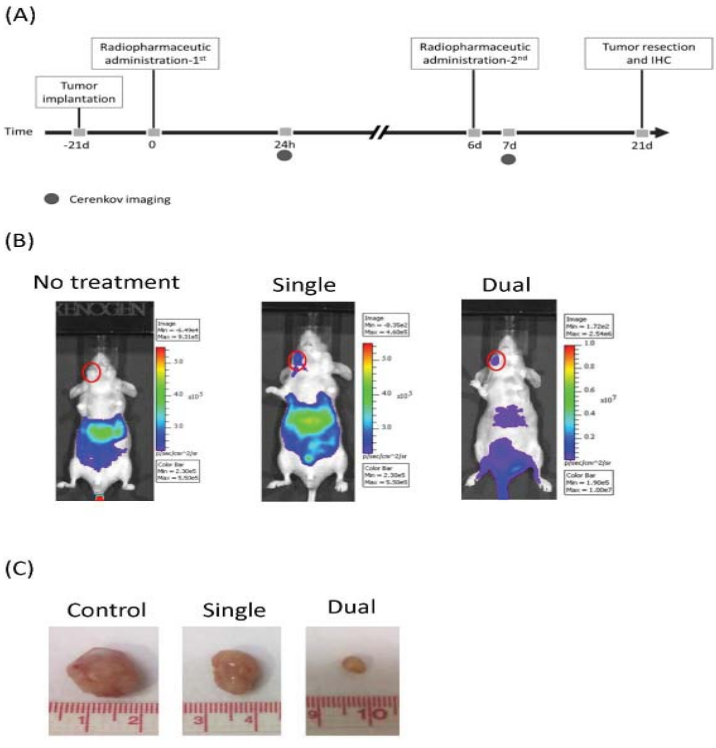


Figure 1: Comparison of dual doses and a single dose of ^{188}Re-liposome on suppression of orthotopic HNSCC tumor model. (A) The experimental scheme of ^{188}Re-liposome treatment and tumor evaluation. (B) Cerenkov luminescent imaging (CLI) of orthotopic tumor treated with ^{188}Re-liposome. (C) Comparison of sizes of the resected tumors from tumor-bearing mice with or without ^{188}Re-liposome treatment.

with a single dose of ^{188}Re-liposome (Figure 1C). These results implied that dual doses of ^{188}Re-liposome would be more effective than a single dose of ^{188}Re-liposome on suppression of tumor growth *in vivo*.

Effects of single dose and dual doses of ^{188}Re-liposome on the expression of Ki67 biomarker

To determine if different effects of tumor suppression by single dose and dual doses of ^{188}Re-liposome was associated with tumor proliferation, the Ki-67 proliferative marker was examined in sections of resected tumors. Compared to the untreated control and the single dose, tumors treated with dual doses of ^{188}Re-liposome expressed a very low level of Ki-67 using IHC staining (Figure 2A). The result was also quantified by IHC scoring (Figure 2B). Therefore, dual doses of ^{188}Re-liposome could better suppress the proliferation of tumor cells *in vivo*.

Effects of single dose and dual doses of ^{188}Re-liposome on the expression of EMT related biomarkers

In addition to the Ki-67 proliferative marker, we also compared the expression of biomarkers associated with EMT mechanism in HNSCC tumors treated with a single dose and dual doses of ^{188}Re-liposome. E-cadherin, vimentin, Snail, Slug, and ZEB-1 were examined, and the results showed that dual doses of ^{188}Re-liposome exhibited stronger effects on suppression of EMT by inducing E-cadherin, and inhibiting vimentin, Snail and Slug, respectively (Figure 3). ZEB-1 was the only EMT promoting molecule suppressed equally by both single dose and dual doses of ^{188}Re-liposome (Figure 3). According to the expression of these molecules, it suggests that dual doses of ^{188}Re-liposome enhanced the therapeutic efficacy by suppressing tumor proliferation and metastasis concomitantly.

Comparison of microRNA expressive profiles in HNSCC tumors treated with a single dose and dual doses of ^{188}Re-liposome

We have previously found that ^{188}Re-liposome can induce *let-7* microRNA as elucidated from the microarray analysis [12]. Here we further investigated the expressive profiles of microRNA in HNSCC tumors treated with ^{188}Re-liposome using the Taqman® Openarray for human microRNA. The heatmaps of microRNA open arrays overviewed a total of 758 microRNA in HNSCC tumors treated with a single dose of ^{188}Re-liposome, dual doses of ^{188}Re-liposome, and untreated control (Supplementary data 1). We shortlisted a group of the most up-regulated and down-regulated miRNAs who have opposite

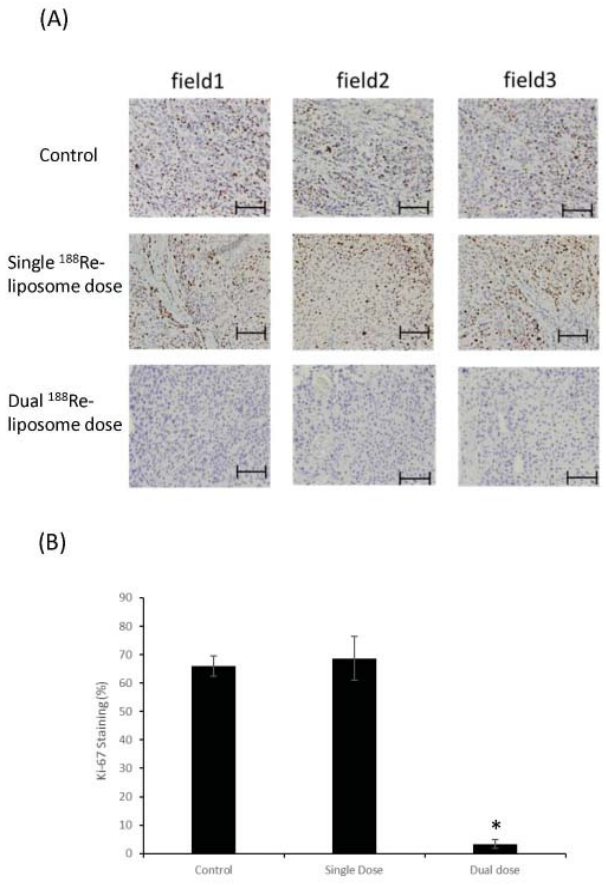


Figure 2: Comparison of Ki-67 proliferative marks in HNSCC tumor sections. (A) IHC staining of Ki-67 in tumor sections with or without the treatment of ^{188}Re-liposome. Three random fields were selected for imaging acquisition and quantification. Scale bar: 100 m. (B) Quantification of IHC staining of Ki-67 markers in tumor treated with different regimes of ^{188}Re-liposome. *: $p < 0.05$.

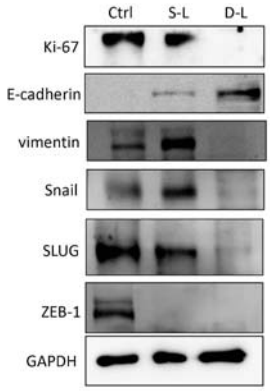
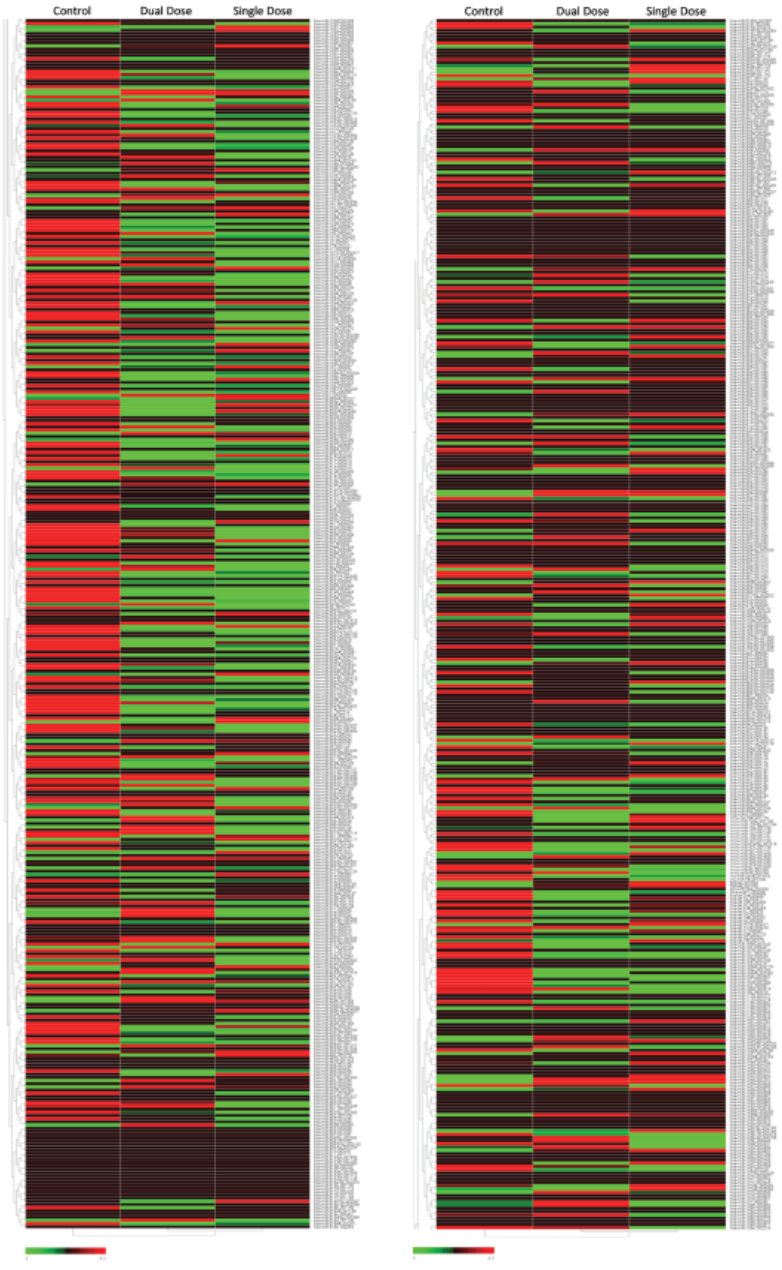


Figure 3: Effects of ^{188}Re-liposome on the expression of EMT related biomarkers. Western blot analysis was used to detect the protein levels of these markers expressed in HNSCC tumors treated with dual doses or a single dose of ^{188}Re-liposome compared to untreated controls.



Supplementary data 1**:** The overall heatmaps of Taqman Openarrays microRNA panel revealed the expressive profiles of 758 microRNA in HNSCC Tumors treated with a single dose of ^{188}Re-liposome, dual dose of ^{188}Re-liposome, and untreated control.

expression profiles between dual doses and a single dose of ^{188}Re-liposome treatment. Cut-off expressive ratios (in log2) between dual doses and a single dose treatment (D/S ratio) were set to be 2 and -1 for up- and down-regulation groups, respectively. Twenty-two microRNAs (miRBase ID: miR-181c-5p, miR-338-5p, miR-1285-3p, miR-146a-5p, miR-25-5p, miR-1225-3p, miR-147b, miR-28-3p, miR-10b-3p, miR-99b-3p, miR-577, miR-361-3p, miR-1260a, miR-150-5p, miR-148b-3p, miR-186-5p, miR-543, miR-592, miR-501-3p, miR-23b-3p, miR-766-3p, and miR-342-3p) were up-regulated by dual doses of ^{188}Re-liposome but concomitantly down-regulated by a single dose of ^{188}Re-liposome (Figure 4A). On the other hand, nineteen microRNAs (miR-872, miR-200a-5p, miR-1267, miR-296-5p, miR-584-5p, miR-29a-5p, miR-200c-5p, miR-1233-5p, miR-let-7i-3p, miR-21-3p, miR-522-3p, miR-629-5p, miR-18a-3p, miR-520e, miR-224-5p, miR-200b-5p, miR-208b-3p, miR-744-5p, and miR-551b-5p) were down-regulated by dual doses of ^{188}Re-liposome accompanied by up-regulation with a single dose of ^{188}Re-liposome (Figure 4B). These two microRNA groups were separately subjected to the KEGG pathway analysis. Most of the genes affected by microRNAs that were up-regulated or down-regulated by dual doses of ^{188}Re-liposome were associated with pathways in cancer as well as other cancer-associated pathways (Table 1 and Table 2). These results suggest that ^{188}Re-liposome would regulate the expression of microRNA in HNSCC and ablate tumor progression.

Demonstration of potent microRNA regulated by single dose and dual doses of ^{188}Re-liposome

According to the KEGG pathway analysis by the microRNA openarray dataset, the intracellular signaling pathways of HNSCC tumors influenced by dual doses of ^{188}Re-liposome were ranked by p - value. Thirty-nine and 62 pathways were significantly affected by the microRNAs down-regulated and up-regulated by dual doses of ^{188}Re-liposome, respectively ($p < 0.05$). According to the rank of p - value, the top 10 signaling pathways affected by miRNAs that were down-regulated or up-regulated by dual doses of ^{188}Re-liposome were different and were shown in table 3 and table 4. Compared to other microRNAs down-regulated by dual doses of ^{188}Re-liposome, miR-520e and miR-522-3p affected most of the genes involved in that top 10 pathways (Table 3). On the other hand, miR-186-5p and miR-543 were involved in

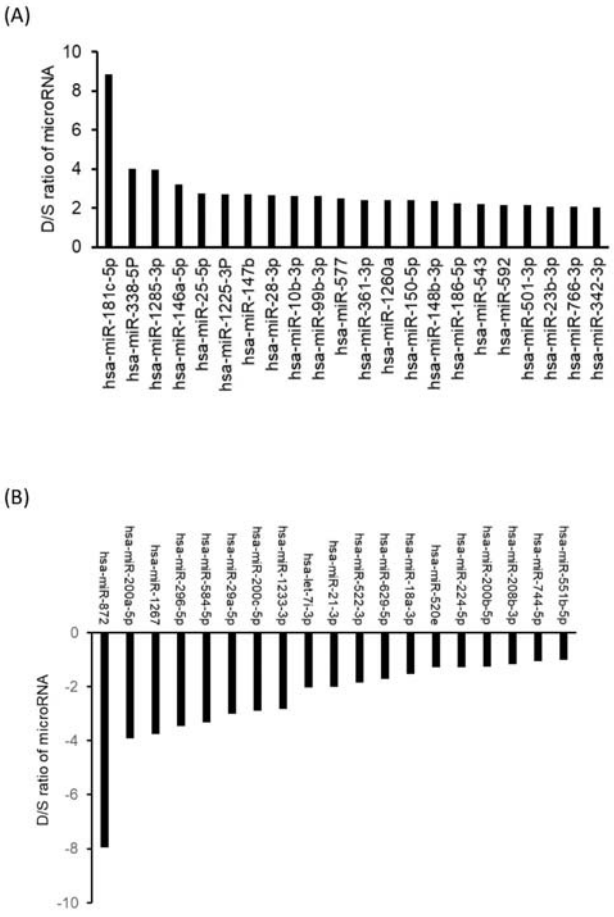


Figure 4: Comparison of microRNAs inversely regulated by different treatment regimes of ^{188}Re-liposome on the orthotopic HNSCC tumor model. (A) The microRNAs up-regulated by dual doses and concomitantly down-regulated by a single dose of ^{188}Re-liposome. (B) The microRNAs down-regulated by dual doses and concomitantly up-regulated by a single dose of ^{188}Re-liposome. The differences between dual doses and single dose of ^{188}Re-liposome regulated microRNA expression (log_2) were represented by D/S ratio of microRNA.

Table 1: Top 10 pathways associated with miRNAs down-regulated by dual doses but up-regulated by single dose of ^{188}Re-liposome

KEGG pathway	#genes	#miRNAs	*p* - value
Pathways in cancer	107	14	0.03296646
MAPK signaling pathway	81	15	0.00192409
Proteoglycans in cancer	67	14	6.89E-08
Ras signaling pathway	63	15	0.01942799
Rap1 signaling pathway	60	12	0.01508721
Transcriptional misregulation in cancer	55	14	0.02290762
Hippo signaling pathway	50	11	6.89E-08
Protein processing in endoplasmic reticulum	47	12	0.0434422
Signaling pathways regulating pluripotency of stem cells	46	15	0.00030753
Dopaminergic synapse	45	12	0.00809897

Table 2: Top 10 pathways associated with miRNAs up-regulated by dual doses but down-regulated by single dose of ^{188}Re-liposome.

KEGG pathway	#genes	#miRNAs	*p* - value
Pathways in cancer	175	21	0.025728198
PI3K-Akt signaling pathway	153	21	0.002721846
MAPK signaling pathway	120	19	0.002721846
Ras signaling pathway	104	19	0.002721846
Regulation of actin cytoskeleton	102	19	0.010643911
Rap1 signaling pathway	101	21	0.001949161
Proteoglycans in cancer	100	19	0.00015307
Focal adhesion	97	19	0.009082868
Endocytosis	94	18	0.018303813
cAMP signaling pathway	90	20	0.029635054
cGMP-PKG signaling pathway	82	20	0.002742686

another 10 signaling pathways mostly affected by dual doses of ^{188}Re-liposome up-regulated miRNAs (Table 4). We next compared the downstream genes regulated by miR-520e and miR-522-3p and found that *SMAD2*, *FZD3*, *PIK3CA,* and *JAK1* genes were involved in these two microRNAs (Figure 5A). For miR-186-5p and miR-543, 13 genes including *FGF12, SMAD2, CBL, PTCH1, TPR, CDK6, FZD3, HDAC2. PIAS2, PIK3CA, PTEN, CREBBP,* and *XIAP* were co-regulated (Figure 5B). Surprisingly, *SMAD2*, *FZD3*, and *PIK3CA* genes were commonly regulated by these four miRNAs, even though miR520e and miR-522-3p were down-regulated, but miR-186-5p and miR-543 were up-regulated by dual doses of ^{188}Re-liposome. The full list of downstream genes regulated by these four microRNAs was provided (Supplementary data 2).

Association of miRNAs affected by ^{188}Re-liposome and patients survival

We next analyzed the association of miR-520e, miR-522-3p, miR-186-5p, and miR-543 with patients survival using the public on-line Kaplan-Meier Plotter tool (see Materials and Methods). High expression of miR-520e and miR-522-3p was associated with a reduced survival rate, while that of miR-186-5p was marginally associated with an increased survival rate of HNSCC cancer patients (Figure 6). On the other hand, higher expression of miR-543 was also associated with lower survival rate (data not shown). As mentioned above, miR-520e and miR-522-3p were suppressed by dual doses of ^{188}Re-liposome treatment, but miR-186-5p was up-regulated accordingly. Hence, it may suggest that these microRNAs could be used as prognostic factors for HNSCC patients treated with ^{188}Re-liposome.

Table 3: Dual doses of ^{188}Re-liposome down-regulated miRNAs that influence most genes and their associated KEGG pathways.

KEGG pathway	hsa-miR-520e (774 genes in db)			hsa-miR-522-3p (1069 genes in db)		
	genes involved	% Involvement	% target genes in db	genes involved	% Involvement	% target genes in db
Hippo signaling pathway	9	18%	1.163%	23	46%	2.152%
Proteoglycans in cancer	18	27%	2.326%	18	27%	1.684%
Lysine degradation	6	33%	0.775%	4	22%	0.374%
TGF-beta signaling pathway	11	41%	1.421%	10	37%	0.935%
Signaling pathways regulating pluripotency of stem cells	13	28%	1.680%	17	37%	1.590%
Mucin type O-Glycan biosynthesis	2	20%	0.258%	4	40%	0.374%
N-Glycan biosynthesis	2	15%	0.258%	2	15%	0.187%
Morphine addiction	3	11%	0.388%	8	30%	0.748%
Glioma	6	25%	0.775%	6	25%	0.561%
MAPK signaling pathway	23	28%	2.972%	23	28%	2.152%

Table 4: Dual doses of ^{188}Re-liposome up-regulated miRNAs that influence most genes and their associated KEGG pathways.

KEGG pathway	hsa-miR-186-5p (1649 genes in db)			hsa-miR-543 (1556 genes in db)		
	genes involved	% Involvement	% target genes in db	genes involved	% Involvement	% target genes in db
Prion diseases	6	38%	0.364%	3	19%	0.193%
ECM-receptor interaction	7	18%	0.424%	5	13%	0.321%
Adrenergic signaling in cardiomyocytes	29	36%	1.759%	18	23%	1.157%
Hippo signaling pathway	26	37%	1.577%	22	31%	1.414%
Sphingolipid signaling pathway	19	29%	1.152%	19	29%	1.221%
Adherens junction	12	27%	0.728%	15	33%	0.964%
Thyroid hormone signaling pathway	20	32%	1.213%	19	30%	1.221%
Estrogen signaling pathway	18	38%	1.092%	13	28%	0.835%
Glioma	17	47%	1.031%	12	33%	0.771%
Amphetamine addiction	21	54%	1.273%	16	41%	1.028%

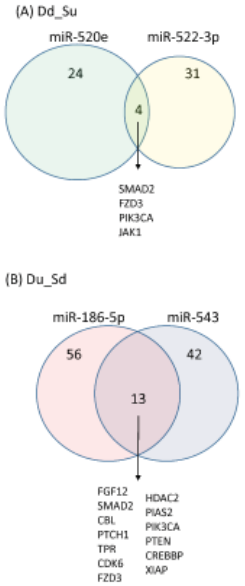


Figure 5: The Venn diagram of different microRNAs regulated downstream genes regulated by different regimes of ^{188}Re-liposome. (A) A collection of downstream genes from miR-520e and miR-522-3p down-regulated by dual doses of ^{188}Re-liposome. (B) A collection of downstream genes from miR-186-5p and miR-543 down-regulated by dual doses of ^{188}Re-liposome.

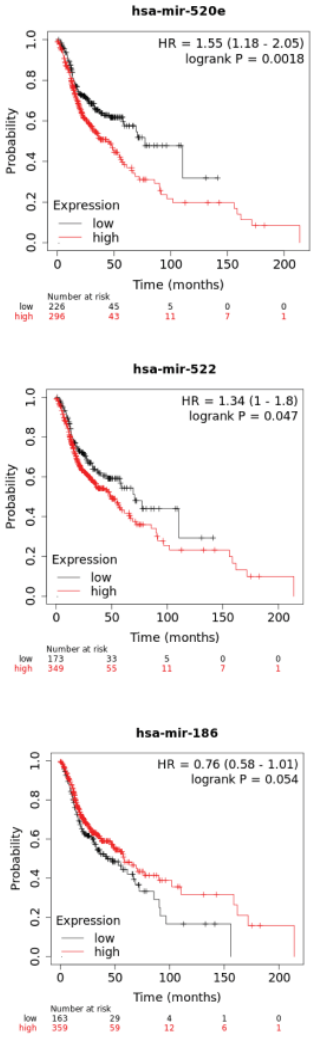
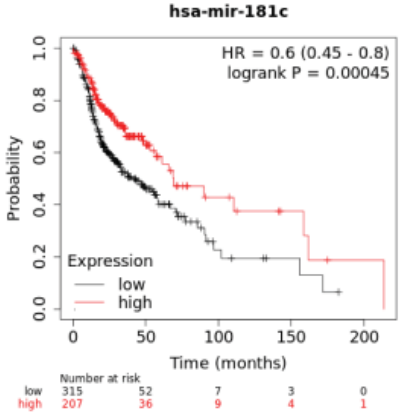


Figure 6: The survival analysis of ^{188}Re-liposome regulated microRNAs using HNSCC patient database. Comparison of high and low expression of miR-520e, miR-522, and miR-186 on overall survivals of on HNSCC patient using the Kaplan-Meier statistics with a log-rank test. $p < 0.05$ represented a significant difference.

DdSu		DuSd	
miR-520e	miR-522-3p	miR-186-5p	miR-543
STAT3	FGF12	FZD7	BRAF
E2F1	PRKCA	FGF12	FGF12
PTGER4	NFKB1	GSK3B	FOS
CXCL8	FGF14	PRKCA	WNT16
SMAD2	SMAD2	FZD5	ITGB1
CRK	CBL	ROCK1	GNA12
PTCH1	HDAC1	FGF14	SMAD2
ROCK2	TCF7L2	SMAD2	CBL
FGF10	WNT5A	CBL	NRAS
F2RL3	CDKN1B	STK4	CRK
FZD3	IGF1R	RUNX1	PIK3CB
MAPK9	FZD3	PTCH1	PTCH1
EDNRB	FZD4	TPR	TPR
SOS1	JUN	HHIP	TCF7L2
PRKCB	SMAD4	GNG12	TGFA
PIAS2	E2F3	WNT2B	HHIP
PIK3CA	CEBPA	RALBP1	COL4A5
LEF1	GLI3	IGF1R	GNA13
FGF16	KIT	EGFR	ROCK2
MAPK1	ITGA2	GNAI3	KRAS
TGFBR2	FZD2	CDK6	CDK6
JAK1	COL4A4	FZD3	FZD3
GNB5	SOS1	PTGER3	CTNNB1
EGLN1	HDAC2	MITF	MAPK8
	NKX3-1	PTK2	GNG2
	CTNNA3	RUNX1T1	PTGS2
	PIK3CA	AR	HDAC2
	EDNRA	PIK3R3	KITLG
	RASGRP3	RASGRP1	PRKCB
	JAK1	EDNRB	IGF1
	XIAP	GLI3	GNAQ
		LPAR5	PIAS2
		COL4A3	PIK3CA
		PIK3R1	HGF
		PIK3CG	PTEN
		HDAC2	FGFR1
		PRKX	MAPK1
		CDC42	CREBBP
		FGF5	FGF7
		PRKCB	TGFBR2
		MAX	BMP4
		IGF1	XIAP
		GNAQ	
		NKX3-1	
		PIAS2	
		PIK3CA	
		MAP2K1	
		LAMC2	
		ITGA6	
		CTNNA2	
		PTEN	
		CREBBP	
		GNAI1	
		PRKACB	
		XIAP	
		PDGFA	

a. DdSu means microRNAs down-regulated by dual dose but up-regulated by single dose of ^{188}Re-liposome. DuSd means an opposite action.

Supplementary data 2:: The downstream genes regulated by 188Re-liposome regulated microRNAs[a].



Supplementary data 3:: Association of miR-181c and HNSCC patient survival.

Discussion

^{188}Re-liposome has been demonstrated to be effective on suppression a variety of human cancers. Most of the studies focused on the biodistribution, tumor targeting, pharmaceutic kinetics and dosimetry previously [9,10,17,22-24]. A phase 0 clinical trial has also shown that ^{188}Re-liposome is a potent theranostic agent for cancer treatment [25]. In this study, we first demonstrated that dual doses of ^{188}Re-liposome exhibited stronger effects than a single dose of ^{188}Re-liposome on suppression of orthotopic HNSCC tumor growth and EMT phenomenon. We have recently showed that repeated treatment of ^{188}Re-liposome on tumor-bearing mice do not cause acute toxicity but reduce blood cell counts [15]. The time interval between the two administrations of ^{188}Re-liposome was over 10 half-lives of this isotope, yet the responses of HNSCC tumors were still enhanced. It is speculated that tumor insulted by first dose of ^{188}Re-liposome has not been fully repaired before the second doses of ^{188}Re-liposome. Notably, the dose of dual treatments were equal and both were at 80% MTD. Thus a sublethal damage repair should not be raised to compromise the efficacy of ^{188}Re-liposome at second treatment.

Most of microRNAs down-regulated by dual doses of ^{188}Re-liposome but up-regulated by a single dose of ^{188}Re-liposome, or vice versa, were associated with genes involved in pathways in cancer according to the KEGG pathway analysis. Although the *p* - value of this category was not smallest in affected pathways (Table 1 and 2), it is convinced that dual doses of ^{188}Re-liposome would vigorously influence microRNA related genes in tumor ablation. Interestingly, the pathway of proteoglycan in cancer accounted for the smallest *p* - value in both conditions of microRNA regulated by dual doses of ^{188}Re-liposome. The signaling of hyaluronan, heparan sulfate proteoglycans, chondroitin sulfate/dermatan sulfate proteoglycan, and keratan sulfate proteoglycan are involved in proteoglycan in cancer pathway, and they are essential for cell adhesion, migration and angiogenesis in KEGG pathway. Indeed, the role of proteoglycan in tumor microenvironment and angiogenesis has been reported [26-28]. Thus, this bioinformatics analysis for microRNA regulation was consistent with the tumor suppressive effects caused by dual doses of ^{188}Re-liposome. The *p* - value of Hippo signaling pathway was the same with proteoglycan in cancer pathway only in microRNAs down-regulated by dual doses of ^{188}Re-liposome. This pathway was even not ranked as primary pathway affected by ^{188}Re-liposome up-regulated microRNAs (Table 2). As Hippo signaling is a critical tumor suppressor pathway, it would be interesting to further investigate how ^{188}Re-liposome regulate this signaling pathway.

According to the data analysis of TaqMan® Openarray Human MicroRNA Panel, miR-520e and miR-522-3p were down-regulated by dual doses of ^{188}Re-liposome but they were also induced by a single dose of ^{188}Re-liposome. On the contrary, miR-186-5p and miR-543 were regulated oppositely by the same regime. Although these microRNAs did not rank as top change by dual doses over a single dose of ^{188}Re-liposome, they influenced most genes in top 10 pathways affected by dual doses of ^{188}Re-liposome. The Venn diagram showed that miR-520e and miR-522-3p co-regulated 4 downstream genes, and miR-186-5p and miR-543 co-regulated 13 downstream genes. Surprisingly, these two groups of microRNAs responded oppositely to dual doses of ^{188}Re-liposome influenced 3 common genes, that is, *SMAD2*, *FZD3*, and *PIK3CA*. *SMAD2* mediates transforming growth factor-β (TGF-β) relayed signaling pathway, and it works differentially with other *SMAD* isoform [29]. TGF-β signaling pathway contains both tumor suppressor and oncogene actions mediated by *SMAD3* [30]. However, several lines of evidence showed that *SMAD2* belongs to tumor suppressor gene of TGF-β signaling pathway [31,32]. Little is known about the function of *FZD3* (Frizzled 3 receptor) gene, although a recent report demonstrated that down-regulation of *FZD3* gene suppresses human melanoma tumorigenesis independent of WNT signaling [33]. *PIK3CA* (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha) gene is also regarded an oncogene, and is usually mutated in cancer cells [34]. It is believed that other genes independently regulated by these two groups of microRNAs are also associated with tumorigenesis. Although we focused on these four microRNAs up-regulated or down-regulated by dual doses of ^{188}Re-liposome, other microRNAs are not excluded and further investigation should be required.

For clinical relevant, we applied the Kaplan-Meier survival analysis to examine if the ^{188}Re-liposome regulated microRNA would be associated with the survival rate of HNSCC patients. For dual doses of ^{188}Re-liposome down-regulated miR-520e and miR-522-3p, both of them exhibit reduced survival rates at high expression. Notably, these two microRNAs were up-regulated by a single dose of ^{188}Re-liposome. Dual doses of ^{188}Re-liposome up-regulated miR-186-5p was associated with enhanced survival rate at high expression, but the significance is margin. MiR-181c-5p was ranked as highest D/S ratio, and this microRNA exhibited significant increase of survival rate at high expression (Supplementary data 3). However, miR-872 had the lowest D/S ratio but the association of this microRNA with survival rate was unavailable using the online KM plot tool. Although current study could not determine the biological significance of these microRNA in modulating the therapeutic efficacy of ^{188}Re-liposome, they might be considered as prognostic factors for different regime of ^{188}Re-liposome treatment.

Conclusion

Current data demonstrated that dual doses of ^{188}Re-liposome exhibited better tumor ablation than a single dose of ^{188}Re-liposome on the orthotopic HNSCC tumor model. We further found that several microRNAs were inversely regulated by these two regimes of ^{188}Re-liposome treatment.

The bioinformatics analysis showed that these microRNAs (41 in total) were mainly involved in cancer related pathways. The specific microRNAs found to be involved in regulation of most of downstream genes in dual doses of ^{188}Re-liposome influenced signaling pathways were associated with survival rates of HNSCC patients based on the public microRNA database. For instance, miR-520e and miR-522-3p down-regulated by dual doses but up-regulated by a single dose of ^{188}Re-liposome were associated with worse survival rate when both of them highly expressed in HNSCC patients. Although the role of these microRNAs on mediating the efficacy of ^{188}Re-liposome on HNSCC tumor remains obscure, they might be used as prognostic factors for evaluating different regimes of ^{188}Re-liposome treatment, at least in part.

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