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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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 further compared the microRNA expressive profile in orthotopic 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-S22-3p 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-S22-3p 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 (\(^{188}\text{Re}\)) 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 \(^{188}\text{Re}\) 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 \(^{188}\text{Re}\) (\(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-liposome would also influence the microRNA expressive profile.

Although \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-liposome. The interested microRNAs were also subjected to survival analysis. The results of \(^{188}\text{Re}\)-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% CO₂ and passaged every two days.

Preparation of Rhenium-\(^{188}\text{Re}\)-liposomal drug

The procedures of \(^{188}\text{Re}\)-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 10⁶) 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 \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-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

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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 188Re-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 188Re-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 188Re-liposome on the growth of orthotopic HNSCC tumor model**

The regimen of 188Re-liposome treatment and the timeline of imaging evaluation and tumor resection on HNSCC tumor-bearing mice were schemed (Figure 1A). Because 188Re-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 188Re-liposome treatment showed CLI signals in tumor-bearing mice (Figure 1B). Although dual doses did not exhibit increased accumulation of 188Re-liposome at tumor lesion, the tumor size was apparently smaller than that treated.
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

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**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 μm. (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 expression 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-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...
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another 10 signaling pathways mostly affected by dual doses of $^{188}$Re-liposome affected microRNAs (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 FGFR12, SMAD2, CBL, PITCH1, 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 microRNAs, 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).

### Table 1: Top 10 pathways associated with microRNAs 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      | 0.0080897  |

### Table 2: Top 10 pathways associated with microRNAs 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      | 0.000271846 |
| MAPK signaling pathway        | 120    | 19      | 0.00271846 |
| Ras signaling pathway         | 104    | 19      | 0.00271846 |
| Regulation of actin cytoskeleton | 102  | 19      | 0.01064391 |
| Rap1 signaling pathway        | 101    | 19      | 0.001949161 |
| Proteoglycans in cancer       | 100    | 19      | 0.00015307 |
| Focal adhesion                | 97     | 19      | 0.009082688 |
| Endocytosis                   | 94     | 18      | 0.018303813 |
| cAMP signaling pathway        | 90     | 20      | 0.029635054 |
| cGMP-PKG signaling pathway    | 82     | 20      | 0.002742686 |

### Table 3: Dual doses of $^{188}$Re-liposome down-regulated microRNAs 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) |
|-------------------------------|--------------------------------|----------------------------------|
| 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% |                                  |
| Muco 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 26% 2.152%     |                                  |

### Table 4: Dual doses of $^{188}$Re-liposome up-regulated microRNAs 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) |
|-------------------------------|----------------------------------|--------------------------------|
| Prion diseases                | 6 36% 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%    |                                  |
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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.
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| Supplementary data 2: | The downstream genes regulated by $^{188}$Re-liposome regulated microRNA$^{a}$. |
|----------------------|----------------------------------------------------------------------------------|
| **miR-520e** | **miR-522-3p** | **miR-188-5p** | **miR-543** |
| STAT3 | FGFR2 | TRAF2 | BRAF |
| EZF1 | PRKCA | FGF12 | FGF12 |
| PTGER4 | NFkB1 | GSK3B | FOS |
| CKCL8 | FGFR4 | PRKCA | WNT16 |
| SMAD2 | SMAD2 | FZD5 | ITGB1 |
| CRK | CBL | ROCK1 | GNA12 |
| PTPRC1 | HDAC1 | FGF14 | SMAD2 |
| ROCK2 | TNF | SMAD2 | CBL |
| FGF10 | WNT5A | CBL | NRAS |
| F2RL3 | CDKN1B | STK4 | CRK |
| FZD3 | GSK3B | RUNX1 | PRKCB |
| MAPK9 | FZD8 | PTPCH1 | PTPCH |
| EDNRB | FZD4 | TPR | TPR |
| SOS1 | JUN | HHI | TCF7L2 |
| PRKCB | SMAD4 | GNG12 | TGF |
| PIAS2 | EZF3 | WNT2B | HHI |
| PIK3CA | CEBPA | RABBP1 | COL6A5 |
| LTF1 | G0S1 | G0S1 | GNA13 |
| FGFR1 | KIT | FGFR | ROCK2 |
| MAPK1 | ITGA2 | GNA13 | KRAS |
| TGFB2Q | FZD2 | CDK6 | CDK6 |
| JAK1 | COL4A4 | FZD3 | FZD3 |
| GNA5 | SOS1 | PTGER3 | CTNNB1 |
| EGFR | HDAC2 | MTF | MAPK3 |
| NXK3-1 | PTX2 | GNG2 | GNG2 |
| CTNNB1 | RUNX1 | PTGER2 | PTGER2 |
| PIK3CA | AR | HDAC2 | HDAC2 |
| EDNRB | PIK3C3 | KITLG | KITLG |
| RASGRP3 | PRKCB | PRKCB | PRKCB |
| JAK1 | EDNRB | IGFR | IGFR |
| XIAP | PIK3C | GNAQ | GNAQ |
| LPAR5 | PIAS2 | PIAS2 | PIAS2 |
| COX4A3 | PRKCA | PRKCA | PRKCA |
| PIK3R1 | HGF | PIK3C5 | PTEN |
| HDAC2 | FGFR1 | HDAC2 | FGFR1 |
| PRX | MAPK1 | CDC42 | CREBBP |
| FGFR5 | FGFR5 | FGFR5 | FGFR5 |
| PRKCB | MAX | BMP4 | BMP4 |
| MAX | IG1 | IG1 | IG1 |
| GNAQ | XIAP | XIAP | XIAP |
| NNX3-1 | PIAS2 | PIAS2 | PIAS2 |
| PIK3C | MAP2K1 | LAMC2 | LAMC2 |
| MAP2K1 | ITGAV | CTNNB2 | CTNNB2 |
| PTEN | CREBBP | GNA1 | GNA1 |
| PRKACB | XIAP | XIAP | XIAP |
| PDGF | XIAP | XIAP | XIAP |

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

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

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Discussion


\(^{188}\text{Re}\)-liposome has been demonstrated to be effective on suppression a variety of human cancers. Most of the studies focused on the biodistribution, tumor targeting, pharmacokinetics and dosimetry previously \([9,10,17,22-24]\). A phase 0 clinical trial has also shown that \(^{188}\text{Re}\)-liposome is a potent theranostic agent for cancer treatment \([25]\). In this study, we first demonstrated that dual doses of \(^{188}\text{Re}\)-liposome exhibited stronger effects than a single dose of \(^{188}\text{Re}\)-liposome on suppression of orthotopic HNSCC tumor growth and EMT phenomenon. We have recently showed that repeated treatment of \(^{188}\text{Re}\)-liposome on tumor-bearing mice do not cause acute toxicity but reduce blood cell counts \([15]\). The time interval between the two administrations of \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-liposome has not been fully repaired before the second doses of \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-liposome at second treatment.

Most of microRNAs down-regulated by dual doses of \(^{188}\text{Re}\)-liposome but up-regulated by a single dose of \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-liposome. This pathway was even not ranked as primary pathway affected by \(^{188}\text{Re}\)-liposome up-regulated microRNAs (Table 2). As Hippo signaling is a critical tumor suppressor pathway, it would be interesting to further investigate how \(^{188}\text{Re}\)-liposome regulate this signaling pathway.

According to the data analysis of TaqMan\textsuperscript{®} Openarray Human MicroRNA Panel, miR-520e and miR-522-3p were down-regulated by dual doses of \(^{188}\text{Re}\)-liposome but they were also induced by a single dose of \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-liposome, they influenced most genes in top 10 pathways affected by dual doses of \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-liposome influenced 3 common genes, that is, SMAD2, FZD3, and PIK3CA. SMAD2 mediates transforming growth factor-\(\beta\) (TGF-\(\beta\)) relayed signaling pathway, and it works differentially with other SMAD isoform \([29]\). TGF-\(\beta\) 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-\(\beta\) 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.

For clinical relevant, we applied the Kaplan-Meier survival analysis to examine if the \(^{188}\text{Re}\)-liposome regulated microRNA would be associated with the survival rate of HNSCC patients. For dual doses of \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-liposome. Dual doses of \(^{188}\text{Re}\)-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 \(^{188}\text{Re}\)-liposome, they might be considered as prognostic factors for different regime of \(^{188}\text{Re}\)-liposome treatment.

Conclusion

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