Radiosensitization effect of hsa-miR-138-2-3p on human laryngeal cancer stem cells

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Background Treatment that targets to cancer stem cells plays an important role in controlling and eliminating of tumor initiation, development, progression, and chemotherapy/radiotherapy resistance. In our previous study, we cultured and harvested human laryngeal cancer stem cells (CSCs), applied microRNA biochips to screen differentially expressed miRNAs that were related to radiation tolerance in irradiated human laryngeal CSCs. According to the predicted genes and pathways of differential miRNAs target, hsa-miR-138-2-3p that was down-regulated expression under radiation, was thought to play a key role in enhancing the radio-sensitivity in human laryngeal squamous cancer stem cells. Method To investigate the radiational enhancement of hsa-miR-138-2-3p, we transfected hsa-miR-138-2-3p mimics that were synthesized based on the sequences of hsa-miR-138-2-3p in vitro into human laryngeal CSCs (Hep-2, M2e, and TU212 cell lines) to make hsa-miR-138-2-3p overexpressed, and the tumorous specialities of CSCs, like cell proliferation, invasion, apoptosis, cell cycle arrest, and DNA damage were evaluated by CCK-8 assay, clone formation assay, invasion assay, flow cytometry, and comet assay. Furthermore, we explored the signal transduction pathways that regulated the cancer stem cell initiation, development, invasion, apoptosis and cell cycle arrest, which were controlled by hsa-miR-138-2-3p. Result Overexpressed hsa-miR-138-2-3p played an key role in many anti-cancer biological process in human laryngeal CSCs: (1) decreased laryngeal CSCs proliferation and invasion in response to radiotherapy; (2) increased the proportion of early and late apoptosis in laryngeal CSCs after radiation, raised G1 phase arrest in laryngeal CSCs after radiation, and decreased the proportion of S stage cells of cell cycle that were related to radio-resistance in laryngeal CSCs; (3) down-regulated the expression of β-catenin in Wnt signal pathway that was related to the
tolerance of laryngeal CSCs to radiotherapy; (4) down-regulated the expression of YAP1 in Hippo signal pathway that regulated cell proliferation, invasion and apoptosis; (5) up-regulated the expression of p38 and JNK1 in MAPK signal pathway that was concerned to radio-sensitivity. **Conclusion** In the present study, it was found that hsa-miR-138-2-3p regulated Wnt/β-catenin pathway, Hippo/YAP1 pathway, and MAPK/p38/JNK1 pathway that were involved in cell proliferation, invasion, apoptosis, cell cycle arrest, and radio-resistance and radio-sensitivity in laryngeal CSCs. These results will be useful for a better understanding of cell biology of hsa-miR-138-2-3p in laryngeal CSCs, and serving hsa-miR-138-2-3p as a promising biomarker and target for diagnosis and novel anti-cancer therapy for laryngeal cancers.
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Abstract

Background Treatment that targets to cancer stem cells plays an important role in controlling and eliminating of tumor initiation, development, progression, and chemotherapy/radiotherapy resistance. In our previous study, we cultured and harvested human laryngeal cancer stem cells (CSCs), applied microRNA biochips to screen differentially expressed miRNAs that were related to radiation tolerance in irradiated human laryngeal CSCs. According to the predicted genes and pathways of differential miRNAs target, hsa-miR-138-2-3p that was down-regulated under radiation, was thought to play a key role in enhancing the radio-sensitivity in human laryngeal squamous cancer stem cells.

Method To investigate the radiational enhancement of hsa-miR-138-2-3p, we transfected hsa-miR-138-2-3p mimics that were synthesized based on the sequences of hsa-miR-138-2-3p in vitro into
human laryngeal CSCs (Hep-2, M2e, and TU212 cell lines) to make hsa-miR-138-2-3p overexpressed, and the tumorous specialities of CSCs, like cell proliferation, invasion, apoptosis, cell cycle arrest, and DNA damage were evaluated by CCK-8 assay, clone formation assay, invasion assay, flow cytometry, and comet assay. Furthermore, we explored the signal transduction pathways that regulated the cancer stem cell initiation, development, invasion, apoptosis and cell cycle arrest, which were controlled by hsa-miR-138-2-3p.

**Result** Overexpressed hsa-miR-138-2-3p played an key role in many anti-cancer biological process in human laryngeal CSCs: (1) decreased laryngeal CSCs proliferation and invasion in response to radiotherapy; (2) increased the proportion of early and late apoptosis in laryngeal CSCs after radiation, raised G1 phase arrest in laryngeal CSCs after radiation, and decreased the proportion of S stage cells of cell cycle that were related to radio-resistance in laryngeal CSCs; (3) down-regulated the expression of β-catenin in Wnt signal pathway that was related to the tolerance of laryngeal CSCs to radiotherapy; (4)down-regulated the expression of YAP1 in Hippo signal pathway that regulated cell proliferation, invasion and apoptosis; (5)up-regulated the expressions of p38 and JNK1 in MAPK signal pathway that was concerned to radio-sensitivity.

**Conclusion** In the present study, it was found that hsa-miR-138-2-3p regulated Wnt/β-catenin pathway, Hippo/YAP1 pathway, and MAPK/p38/JNK1 pathway that were involved in cell proliferation, invasion, apoptosis, cell cycle arrest, and radio-resistance and radio-sensitivity in laryngeal CSCs. These results will be useful for a better understanding of cell biology of hsa-miR-138-2-3p in laryngeal CSCs, and serving hsa-miR-138-2-3p as a promising biomarker and target for diagnosis and novel anti-cancer therapy for laryngeal cancers.

**Introduction**

Many studies have shown that biological characteristics of tumors, including radiation tolerance, are determined by tumor stem cells. Rycaj et al[1] showed that breast cancer and malignant glioma were tolerant to radiotherapy mainly due to the existence of cancer stem cells, which had powerful DNA repair capacity, defensive ability against reactive oxygen species and strong self-renewal capacity.
Claudia et al[2] indicated that the plasticity of cancer stem cells would be beneficial under the stress of various factors, and cancer stem cells (CSCs) were involved in reducing radio-sensitivity by changing their structures to generate radiation-induced resistance. Selcuk et al[3] found that the nature of radiation resistance of tumor cells were cancer stem cells that prevented cancer cell apoptosis and enhanced the efficiency of DNA repair. Therapies target to CSCs are necessary for controlling and eliminating tumor cell growing, invasion, progression and radio-resistance. Yosuke et al [4] found that the removal of Brachyury that was related to cell proliferation, migration, invasion, radiation and chemotherapy resistance of Adenoid Cystic carcinoma CSCs, could improve tumor stem cell radiation sensitivity. Wu et al[5] developed a model of CDK1 knock-out brain malignant glioma and found that CDK1 contributed to enhance radiosensitivity of CSCs by inducing cell apoptosis. Cell cycle checkpoint kinases (CDK1 and CDK2) play key roles in DNA damage response in radiation and chemotherapy.

MicroRNA (miRNA), a non-coding oligonucleotide single chain of ~20nt in length, was recently considered as the most important gene regulation factor in cancer cells, and may improve the radiosensitivity of tumor cells[6]. Jiang et al[21] found that increased expression of miR-17-92 cluster could enhance the resistance to radiation of lymphoma. Wu et al[22] demonstrated that MiR-148b could enhance radiosensitivity of non-Hodgkin’s lymphoma cells by promoting apoptosis. However, the role of hsa-miR-138-2-3p in laryngeal CSCs was not reported before.

In our previous study[7], we used microRNA biochips to compare and screen the differential expression microRNAs in laryngeal CSCs in response to radiation stress. Based on the predicted genes and pathways of miRNA target, the expression profile of hsa-miR-138-2-3p that was down-regulated significantly after radiation, was thought to play an important role in regulation of radiosensitivity in laryngeal squamous CSCs. In present study, we synthesized hsa-miR-138-2-3p in vitro and transfected it into three types of laryngeal CSCs (Hep-2, M2e, TU212) to make hsa-miR-138-2-3p overexpressed, and the tumorous specialities of CSCs, such as cell proliferation, invasion, apoptosis, cell cycle arrest, and DNA damage. Furthermore, we explored the signal transduction pathways that
were involved in cell initiation, development, invasion, apoptosis and cell cycle arrest, which were regulated by hsa-miR-138-2-3p. These results will be useful for a better understanding of cell biology of hsa-miR-138-2-3p in laryngeal CSCs, and serving hsa-miR-138-2-3p as a promising biomarker and target for diagnosis and novel anti-cancer therapy for laryngeal cancers.

Materials and Methods

Laryngeal cancer sphere culture

Three human laryngeal squamous cancer cell lines, Hep-2, TU212 and M2e, were obtained from the American Type Culture Collection (ATCC, USA). Serum supplement medium (SSM) contained 90% RPMI-1640 (Gibco, USA) and 10% fetal bovine serum (Gibco, USA). Serum free medium (SFM) contained DMEM/F12 (Gibco, USA); and 4 mg/ml heparin; 10 ng/ml basic fibroblast growth factor (bFGF, Peprotech, USA), 20 ng/ml epidermal growth factor (EGF, Peprotech, USA); 25 mg/ml insulin; and 2ml 50X B27 supplement (Gibco, USA). Cells in exponential growth phase were washed with PBS (Gibco, USA) and digested with 0.25 trypsin/0.02% ethylenediaminetetraacetic acid (EDTA, Gibco, USA), followed by resuspension in SFM at a concentration of 5X10E5 cells/ml. The medium was changed every 5 days in half amount. Each cell line was regularly observed to confirm its morphology and absence of mycoplasma contamination.

Sorting of laryngeal CSCs based on cell surface marker expression

The laryngeal cancer sphere of Hep-2, M2e and TU212, was digested, a single-cell suspension was prepared and the cell number was counted before labeling. Cells were collected by centrifuge at 1000 rpm for 5 min and the cell pellets were resuspended in 90ul of PBS buffer per 10E7 total cells. 10ul of anti-human-CD133-FITC (AC-133-FITC, mouse IgG1, Miltenyi, Germany) were added. The samples were mixed well and incubated in the dark for 30 min at 4°C refrigerator. The analysis was performed with FACS caliber (BD, USA), and CD133 positive expression cells were investigated as laryngeal CSCs.

Hsa-miR-138-2-3p targets prediction

In our earlier research [7], laryngeal CSCs were harvested and accepted to radiation stress.
applied microRNA biochips to identify and screen differential expression miRNAs, and more than 2-fold up-regulation/down-regulation expression were considered as differential expressions.

Meaningful miRNAs were selected by targeted genes from Targetscan Human 6.2 (http://www.targetscan.org [10]) and miRanda (http://www.microrna.org/microrna/home.do [11]). The sequences of miRNAs were inquired from miRBase (http://www.mirbase.org [12]). To understand the targeted biological process, we applied starBase v2.0 (http://starbase.sysu.edu.cn/index.php) [13] to analyze signal transduction pathways that were regulated by microRNAs from pathway databases (e.g. GO, KEGG, BIOCARTA). Hsa-miR-138-2-3p mimics, nonsense oligonucleotides, and negative control FAM oligonucleotides with fluorescence were synthesized in vitro (invitrogen, Shanghai, China).

**Transient cell transfection**

Laryngeal CSCs (2X10E5 cells/ well) were plated in 12-well culture plates, and were transfected equal volume with gradient concentrations of hsa-miR-138-2-3p mimics (conc: 50 nM, 100 nM, 150 nM). Nonsense oligonucleotides (conc: 100 nM), negative control FAM oligonucleotides (conc: 100 nM), and PBS buffer with the same volume as hsa-miR-138-2-3p were transfected into laryngeal CSCs. The hsa-miR-138-2-3p teams with gradient concentration were considered as experimental team and were named as 50nM-TR, 100nM-TR, 150nM-TR respectively. Nonsense oligonucleotides team, negative control FAM oligonucleotides team, and PBS buffer team were considered as control teams, and were named as 100nMN-CR, FAM-CR, and PBS-CR. All the teams were added in Entranster™-R transfection reagent (Engreen Biosystem, China) and mixed sufficiently, according to the manufacturer’s instructions. All teams were with the final concentrations of 50nM per well. After mixing, all 12-well culture plates were incubated for 6h at 4°C refrigerator. The transfection efficiency of hsa-miR-138-2-3p mimics and nonsense oligonucleotides were evaluated by the positive expression of negative control FAM oligonucleotides by flow cyometry.

**Irradiation**

Laryngeal CSCs were irradiated by a linear accelerator with a 6-MV X ray. Culture plates were placed
under a 15mm tissue equivalent filler. The distance between filler and radiation source was 100mm.

Experimental teams and control teams were irradiated continuously at total does of 2Gy each day, for 2 days. 100nM-T, 100nMN-C, PBS-C teams were treated without radiation and were placed out the range of radiation at the same time. All the three teams were served as control teams.

**Proliferation assays**

All the experimental and control teams were respectively resuspended in 0.1 ml SFM at a density of 5000 cells/well in 96-well microwell culture plates. Cell proliferation were analyzed at 0h, 24h, 48h and 72h after the second radiation by CCK-8 assay (Cell Counting Kit-8, Engreen Biosystem, China)[14]. Viable cells were quantified by measuring absorbance at 450 nm absorption spectra in a microplate reader, and were named as “A450 value”.

**Apoptosis and cell cycle assay**

For apoptosis assay, 100nM-TR and 100nMN-CR of Hep-2, M2e, and TU212 CSCs were resuspended in PBS buffer by the amount of cells 5000/ml. 195 ul cell suspension were mixed well with 5 ul Annexin V-FITC and incubated at room temperature for 10 min. Cells were washed with PBS and resuspended in 190 ul deliquated binding buffer, then 10 ul 20 ug/ml PI were added. Identified by flow cytometry, cells were divided into four sections: Q1: Annexin V-FITC - PI +, was representative of mechanical error; Q2: Annexin V-FITC+ PI+, was representative of late apoptosis or necrosis cells; Q3: Annexin V-FITC - PI -, was representative of living cells; Q4: Annexin V-FITC+ PI-, was representative of early apoptosis cells.

For cell cycle analysis, 100nM-TR and 100nMN-CR of Hep-2, M2e, and TU212 CSCs were washed with cold PBS for three times, and then the cells were fixed in 70% ethanol at -20°C for 12h. Following the fixation, cells were washed with cold PBS and stained with 500ul PI at 37°C in dark for 30min. Analyses were performed on flow cytometry.

**Invasion assays**

For Transwell migration assay, 12-well Transwell chambers containing 8 um pores were coated with 700 ul Matrigel (BD, USA) at -20°C. According to the manufacturer’s instructions[15], 100nM-TR and
100nMN-CR of Hep-2, M2e, and TU212 CSCs were collected and resuspended in 1 ml DMEM/F12 at a density of 2X10E5 cells/ml. 1 ml cell suspension of six teams were added to the upper Transwell chamber, and 600 ul SFM were added to the lower chamber. After 24h of cell incubation, cells that were cultured previously above the upper chambers, migrated to the other side of the upper chambers. The reverse side of the upper chambers were fixed with 4% paraformaldehyde and stained with crystal violet. The numbers of migrated cells on the reverse side were counted at least five random microscopic fields by a light microscope at a magnification of 200X (Olympus, Japan).

**Clone formation assay**

100nM-TR, 100nMN-CR, and non-transfection teams of Hep-2, M2e, and TU212 CSCs that proliferated at exponential growth phase, were digested and cultured at the density of 10E6/dish in 10 cm petri dishes. After 24h of culture, cells were treated with 0, 2, 4, 6, 8 Gy X-ray irradiation respectively, and then were incubated for additional 14 days. After incubation, cells were washed with PBS buffer for 3 times, and fixed with paraformaldehyde for 20 min and air dried overnight. Cells were then washed with water and the colonies (> 50 cells) were counted for each culture dish by a light microscope (Olympus, Japan).

**Comet assay**

100nM-TR and 100nMN-CR of Hep-2, M2e, and TU212 CSCs at equal volume of cell suspension (4X10E5 cells) were mixed with 0.5% (w/v) low melting agarose (LMA) in 0.01 M PBS buffer respectively. The mixture were pipetted on the frosted slides with pre-coating of normal melting agarose 1% (w/v). After the agarose solidified, another 100 μl of 0.5% (w/v) LMA were pipetted on the slides and immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl buffer, 0.1% SDS and 1% Triton X-100 and 10% DMSO; pH 10.0) for 120 min in dark at 4°C to lyse the cellular and nuclear membranes. The slides were rinsed with unwinding buffer and transferred into an electrophoresis tank containing unwinding buffer (3 M NaOH, 10 mM EDTA; pH 13.0) for denaturing the DNA followed by electrophoresis for 30 min with an electric current of 25 V. The slides were washed twice with neutralizing buffer (0.4 M Tris-HCl; pH 7.5) for 10 min and ethanol treatment was...
done another 5 min. Ethidium bromide (20 mg/ml) 40 μl were used to stain the slides and DNA damage visualized using fluorescence microscope (Olympus, Japan). Appearance of 'comet' with fragmented DNA (tail) being separated from undamaged nuclear DNA (head) was seen in damaged cells and measurements were made by Comet Assay IV software to determine the tail movement (%). The results were expressed as percent tail movement.

**Western blotting**

The total protein of 100nM-TR, 100nMN-CR, and non-transfection teams of Hep-2, M2e, and TU212 CSCs were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes. The membranes were blocked in 5% BSA, diluted in 1X TBS-Tween for 1h and then incubated overnight with anti-human β-catenin/YAP1/p38/JNK1 antibody (Abcam, USA) according to the manufacturer’s instructions. Primary antibody binding was detected with secondary IgG-HRP antibodies goat anti-rabbit (Abcam, USA). Actin was used as control. Images were captured on MicroChemi 4.2 (Eastwin, Israel).

**Statistical analysis**

Data are shown as mean ± standard deviation (±s). One-way and two-way ANOVA analyses were applied to compare the sample means of test groups and control groups. The Fisher’s exact test was applied to compare the sample rates of test groups and control groups. All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., USA). P<0.05 was considered statistically significant.

**Results**

1. **Hsa-miR-138-2-3p was selected to transfected into the laryngeal CSCs**

Based on our previous study [7], we applied Targetscan and Miranda to investigate target genes of hsa-miR-138-2-3p, such as MAP3K11, CASP3 and HIF1AN, Which were involved in cell apoptosis, radio-sensitivity, and cell cycle arrest (Table 1).

**Table 1. Target genes of hsa-miR-138-2-3p**
| Target gene | Representative transcript | Gene name |
|-------------|---------------------------|-----------|
| MAP3K11     | NM_002419                 | mitogen-activated protein kinase kinase kinase 11 |
| ARHGEF3     | NM_001128615              | Rho guanine nucleotide exchange factor (GEF) 3 |
| HIF1AN      | NM_017902                 | hypoxia inducible factor 1, alpha subunit inhibitor |
| CASP3       | NM_004346                 | caspase 3, apoptosis-related cysteine peptidase |
| ACVR2B      | NM_001106                 | activin A receptor, type IIB |

The sequences of hsa-miR-138-2-3p were inquired from miRBase, and hsa-miR-138-2-3p mimics, nonsense oligonucleotides, and negative control FAM oligonucleotides with fluorescence were synthesized in vitro (invitrogen, Shanghai, China). The oligonucleotide sequences were listed in Table 2.

### Table 2. Sequence of synthesized oligonucleotides *in vitro*

| Oligonucleotides             | Sequence                      |
|------------------------------|-------------------------------|
| hsa-miR-138-2-3p mimics      | 5’ - GCUAUUUUCACGACACCAGGGUU - 3’ |
| nonsense oligonucleotides    | 5’-AAGGCAAGCUGACCCUGAAGU-3’    |
|                              | 3’ - UUCAGGGUCAGCUUGCCUUU- 5’ |
| Fluorescein FAM tag oligonucleotides | 5’-UUCUCCGAACGUGUCACGUTT-3’ |
|                              | 3’ - ACGUGACACGUCCGAGAATT- 5’ |

To evaluate the transfection efficiency of hsa-miR-138-2-3p and nonsense oligonucleotides, the FAM-CR teams of Hep-2, M2e, and TU212 cell lines that were transfected with FAM-labeled oligonucleotide were digested and resuspended in PBS buffer. The percentages of FAM-positive cells were identified by flow cytometry. As shown in Figure 1, the transfection efficiency of Hep-2, M2e, and TU212 cell lines were 82.9%, 91.5% and 90.5% respectively, and it was indicated that hsa-miR-138-2-3p and nonsense oligonucleotides were successfully transfected into the laryngeal CSCs with high efficiency.
Figure 1. Cell transfection efficiency evaluated by Flow Cytometry.

Figure 1A, 1B and 1C showed the transfection efficiency of FAM-CR teams of Hep-2, M2e, and TU212 cell line respectively, and the percentages of FAM-positive cells (represented by “P2”) were identified by flow cytometry. The transfection efficiency of Hep-2, M2e, and TU212 cell lines were 82.9%, 91.5% and 90.5% respectively, and it was indicated that hsa-miR-138-2-3p and nonsense oligonucleotides were successfully transfected into the laryngeal CSCs with high efficiency.

2. Overexpressed hsa-miR-138-2-3p was involved in various biological process to enhance the radiosensitivity of laryngeal CSCs

2.1. Overexpressed hsa-miR-138-2-3p inhibited cell proliferation after radiation

The cell proliferation rates of each team at 0, 24, 48 and 72 hours after radiation were shown in Figure 2. From Figure 2A, we can infer that at 48h after radiation, the cell proliferation rate of 50nM-TR, 100nM-TR, and 150nM-TR were lower than 100nMN-CR and PBS-CR. But at 0h, 24h and 72h after radiation, the difference were not observed (P>0.05). It was noted that at 48h after radiation, the cell proliferation rate of 50nM-TR, 100nM-TR, and 150nM-TR were slower than 100nMN-CR and PBS-CR, the differences were statistically significant (P<0.001, P<0.001, P<0.001), and the inhibition capacity of 100nM-TR and 150nM-TR were stronger than 50nM-TR. While, from Figure 2B, it showed that without radiation, the differences of cell proliferation rate of 100nM-T, 100nMN-C, and PBS-C were not statistically significant (P>0.05).
Figure 2. Overexpressed hsa-miR-138-2-3p inhibit cell proliferation after radiation. Figure 2A showed the comparison of cell proliferation among 50nM-TR, 100nM-TR, 150nM-TR, 100nMN-CR and PBS-CR of Hep-2 cell line after radiation. Figure 2B showed the comparison of cell proliferation among 100nM-T, 100nMN-C and PBS-C of Hep-2 cell line without radiation. The vertical and horizontal axis standed for A450 (absorbance at 450 nm absorption spectra) value and time after radiation respectively. From Figure 2A, we can infer that at 48h after radiation, the cell proliferation rate of 50nM-TR, 100nM-TR, and 150nM-TR were lower than 100nMN-CR and PBS-CR. But at 0h, 24h and 72h after radiation, the difference were not observed (P>0.05). It was noted that at 48h after radiation, the cell proliferation rate of 50nM-TR, 100nM-TR, and 150nM-TR were slower than 100nMN-CR and PBS-CR, the differences were statistically significant (***P<0.001), and the inhibition capacity of 100nM-TR and 150nM-TR were stronger than 50nM-TR. While, from Figure 2B, it showed that without radiation, the differences of cell proliferation rate of 100nM-T, 100nMN-C, and PBS-C were not statistically significant (P>0.05). Date are reported as mean±SD.

2.2 Overexpressed hsa-miR-138-2-3p induced cell apoptosis after radiation

To investigate whether the declined cell proliferation was due to the cell apoptosis, we used flow cytometry analyses to evaluate the effect of overexpressed hsa-miR-138-2-3p on promotion of cell apoptosis. We found that the proportion of early apoptosis and late apoptosis of Hep-2, M2e, and TU212 CSCs induced by transfection of hsa-miR-138-2-3p were larger than that induced by transfection of nonsense oligonucleotides, the differences between them were statistically significant (P<0.001)(Figure 3, Figure4).
Figure 3. Overexpressed hsa-miR-138-2-3p induced cell apoptosis after radiation by flow cytometry

Figure 3A and 3D showed the cell apoptosis analysis of 100nM-TR and 100nMN-CR of Hep-2 cell line after radiation respectively; Figure 3B and 3E showed the cell apoptosis analysis of 100nM-TR and 100nMN-CR of M2e cell line after radiation respectively; Figure 3C and 3F showed the cell apoptosis analysis of 100nM-TR and 100nMN-CR of TU212 cell line after radiation respectively. The vertical and horizontal axis in the Figure 3 standed for PI positive area and FITC positive area respectively. Identified by flow cytometry, cells were divided into four sections: Q1: Annexin V-FITC - PI +, was representative of mechanical error; Q2: Annexin V-FITC+ PI+, was representative of late apoptosis or necrosis cells; Q3: Annexin V-FITC - PI -, was representative of living cells; Q4: Annexin V-FITC+ PI-, was representative of early apoptosis cells. Figure 3A, 3B, 3C were shown the proportion of early apoptosis (29.6%, 33.6%, 23.5%) and late apoptosis (25.2%, 17.7%, 18.1%) of Hep-2, M2e, and TU212 CSCs induced by transfection of 100nM hsa-miR-138-2-3p were larger than that of induction by transfection of 100nM nonsense oligonucleotides (Figure 3D: ...
early apoptosis of Hep-2 CSCs was 10.8%; Figure 3E: early apoptosis of M2e CSCs was 8.0%; Figure 3F: early apoptosis of TU212 CSCs was 3.7%; Figure 3D: late apoptosis of Hep-2 CSCs was 8.0%; Figure 3E: late apoptosis of M2e CSCs was 6.7%; Figure 3F: late apoptosis of TU212 CSCs was 5.8%) respectively after radiation.

Figure 4. Overexpressed hsa-miR-138-2-3p induced cell apoptosis after radiation

Figure 4A and 4B showed the comparison of early and late apoptosis between 100nM-TR and 100nMN-CR of Hep-2 cell line after radiation respectively; Figure 4C and 4D showed the comparison of early and late apoptosis between 100nM-TR and 100nMN-CR of M2e cell line after radiation respectively; Figure 4E and 4F showed the comparison of early and late apoptosis between 100nM-TR and 100nMN-CR of TU212 cell line after radiation respectively. The vertical and horizontal axis in the Figure 4 standed for cell numbers and early/late apoptosis respectively. We found that the cell numbers of early apoptosis and late apoptosis of Hep-2, M2e, and TU212 CSCs induced by transfection of 100nM hsa-miR-138-2-3p were larger than that induced by transfection of 100nM nonsense oligonucleotides respectively after radiation, the differences between them were statistically significant (*** P<0.001).

2.3 Overexpressed hsa-miR-138-2-3p induced cell cycle arrest after radiation

DNA content stained with PI were detected with flow cytometry, and cell cycle distribution of Hep-2, M2e, and TU212 cell lines were analyzed by ModFit software[17]. As Figure 5 shown, the growth cycle of cell is divided into five stages, known as G0 phase, in which cell is quiescent, G1 and G2 phase, in which it increases in size, S phase, in which it duplicates its DNA, and M phase, in which it undergoes mitosis and divides. As shown in Figure 6 and Figure 7, the percentage of Hep-2, M2e,
and TU212 CSCs induced by transfection of 100nM hsa-miR-138-2-3p in G1 phase (68.68%, 65.95%, 65.24%) were more than that induced by transfection of 100nM nonsense oligonucleotides (55.44%, 56.90%, 59.23%) respectively after radiation. While, the percentage of Hep-2, M2e, and TU212 CSCs induced by transfection of 100nM hsa-miR-138-2-3p in S phase (23.32%, 26.05%, 26.76%) were less than that induced by transfection of 100nM nonsense oligonucleotides (36.56%, 37.25%, 37.96%) respectively after radiation. The percentage of M2e, and TU212 CSCs induced by transfection of 100nM hsa-miR-138-2-3p in G2 phase (8%, 8%) were more than that induced by transfection of 100nM nonsense oligonucleotides (5.85%, 2.81%) respectively after radiation, but the percentage of Hep-2 CSCs induced by transfection of 100nM hsa-miR-138-2-3p in G2 phase (8%) was as the same as that induced by transfection of 100nM nonsense oligonucleotides (8%) after radiation. However, all differences between them were not statistically significant.

Figure 5. Cell division cycle analysis

As shown in Figure 5A,5B, it is cell division cycle. G0 is the abbreviation of Gap0, cell in G0 phase has left the cycle and has stopped dividing, and cell cycle starts with this phase; G1 is the abbreviation of Gap1, cell in G1 phase increase in size, and is ready for DNA synthesis; S is the abbreviation of Synthesis, cell in S phase is for DNA replication; G2 is the abbreviation of Gap2, cell in G2 phase continue growing; M is the abbreviation of Mitosis, cell in M phase is stopped growing, and cellular energy is used for the orderly division into two daughter cells.
Figure 6. Overexpressed hsa-miR-138-2-3p arrested cell cycle at G1/S phase after radiation by flow cytometry

Figure 6A and 6D showed the cell cycle analysis of 100nM-TR and 100nMN-CR of Hep-2 cell line after radiation respectively; Figure 6B and 6E showed the cell cycle analysis of 100nM-TR and 100nMN-CR of M2e cell line after radiation respectively; Figure 6C and 6F showed the cell cycle analysis of 100nM-TR and 100nMN-CR of TU212 cell line after radiation respectively. The vertical and horizontal axis in the Figure 6 stood for cell numbers and cell division cycle respectively. The percentage of Hep-2, M2e, and TU212 CSCs induced by transfection of 100nM hsa-miR-138-2-3p in G1 phase (68.68%, 65.95%, 65.24%) were more than that induced by transfection of 100nM nonsense oligonucleotides (55.44%, 56.90%, 59.23%) respectively after radiation. While, the percentage of Hep-2, M2e, and TU212 CSCs induced by transfection of 100nM hsa-miR-138-2-3p in S phase (23.32%, 26.05%, 26.76%) were less than that induced by transfection of 100nM nonsense oligonucleotides (36.56%, 37.25%, 37.96%) respectively after radiation. The percentage of M2e, and TU212 CSCs induced by transfection of 100nM hsa-miR-138-2-3p in G2 phase (8%, 8%) were higher than that induced by transfection of 100nM nonsense oligonucleotides (5.85%, 2.81%) respectively after radiation, but the percentage of Hep-2 CSCs induced by transfection of 100nM hsa-miR-138-
2-3p in G2 phase (8%) was as the same as that induced by transfection of 100nM nonsense oligonucleotides (8%) after radiation.

**Figure 7. Overexpressed hsa-miR-138-2-3p arrested cell cycle at G1/S phase after radiation**

Figure 7A and 7B showed the cell cycle analysis of 100nM-TR and 100nMN-CR of Hep-2 cell line after radiation respectively; Figure 7C and 7D showed the cell cycle analysis of 100nM-TR and 100nMN-CR of M2e cell line after radiation respectively; Figure 7E and 7F showed the cell cycle analysis of 100nM-TR and 100nMN-CR of TU212 cell line after radiation respectively. The vertical and horizontal axis in the Figure 7 standed for proportions and cell division phases respectively. The proportion of Hep-2, M2e, and TU212 CSCs induced by transfection of 100nM hsa-miR-138-2-3p in G1 phase were higher than that induced by transfection of 100nM nonsense oligonucleotides respectively after radiation. While, the percentage of Hep-2, M2e, and TU212 CSCs induced by transfection of 100nM hsa-miR-138-2-3p in S phase were less than that induced by transfection of 100nM nonsense oligonucleotides respectively after radiation. The percentage of M2e, and TU212 CSCs induced by transfection of 100nM hsa-miR-138-2-3p in G2 phase were more than that induced by transfection of 100nM nonsense oligonucleotides respectively after radiation, but the percentage of Hep-2 CSCs induced by transfection of 100nM hsa-miR-138-2-3p in G2 phase was as the same as that induced by transfection of 100nM nonsense oligonucleotides after radiation. However, all differences between them were not statistically significant (P>0.05).

2.4 Overexpressed hsa-miR-138-2-3p inhibited cell invasion after radiation

Cancer migration and invasion are serious and fatal steps in cancer progression [6,23], so it is
important and urgent to identify therapeutic targets to prevent the metastases of cancer cells. We found that hsa-miR-138-2-3p played a key role in inhibiting laryngeal CSCs invasion. Transwell invasion assay demonstrated that the numbers of Hep-2, M2e, and TU212 CSCs that were transfected into 50nM, 100nM, and 150nM hsa-miR-138-2-3p penetrated through matrigel were lower than that were transfected into 100nM nonsense oligonucleotides and PBS buffer at 24h after radiation, a significant difference between experimental teams and control teams (Figure 8, 9). It was important to note that the cell numbers of 100nM-TR and 150nM-TR were lower than 50nM-TR and the differences were statistically significant. However, this phenomenon was not investigated without radiation, the cell numbers of 100nM-C were not lower than 100nMN-C and PBS-C (Figure 8, 9).

**Figure 8. Overexpressed hsa-miR-138-2-3p inhibited cell invasion by Transwell assay**

Figure 8A, 8B and 8C showed Transwell Assay of 100nM-T, 100nMN-C and PBS-C of Hep-2 cell line without radiation respectively; Figure 8D, 8E, 8F, 8G and 8H showed Transwell Assay of 50nM-TR, 100nM-TR, 150nM-TR, 100nMN-CR and PBS-CR of Hep-2 cell line 24h after radiation respectively. The cell numbers of experimental teams and...
control teams of Hep-2 CSCs were reduced at 24h after radiation. Among them, Hep-2 CSCs were dropped most. The cell number of 50nM-TR, 100nM-TR, and 150nM-TR of Hep-2 CSCs were (121.6±4.62), (41.6±4.62) and (40.8±3.63) were lower than 100nMN-CR (168.80±23.2) and PBS-CR (187.08±24.6) of that respectively. It was important to note that the cell numbers of 100nM-TR and 150nM-TR were lower than 50nM-TR and the differences were statistically significant. However, this phenomenon was not found in non-radiation, the cell numbers of 100nM-T, 100nMN-C, and PBS-C were (212.6±29.9), (226.6±32.2), and (228.2±33.5), respectively.

Figure 9. Overexpressed hsa-miR-138-2-3p inhibited laryngeal CSCs invasion

Figure 9A showed the comparison of Transwell Assay among 100nM-T, 100nMN-C and PBS-C of Hep-2 cell line without radiation respectively; Figure 9B showed the comparison of Transwell Assay of 50nM-TR, 100nM-TR, 150nM-TR, 100nMN-CR and PBS-CR of Hep-2 cell line 24h after radiation respectively. The vertical and horizontal axis in the Figure 9A and 9B stood for cell numbers and treatments respectively. Transwell invasion assay demonstrated that the numbers of Hep-2 CSCs that were transfected into 50nM, 100nM, and 150nM hsa-miR-138-2-3p penetrated through matrigel were lower than that were transfected into 100nM nonsense oligonucleotides and PBS buffer at 24h after radiation, a significant difference between experimental teams and control teams (***P<0.001) (Figure 9B). It was important to note that the cell numbers of 100nM-TR and 150nM-TR were lower than 50nM-TR and the differences were statistically significant (**P<0.001). However, this phenomenon was not investigated without radiation, the cell numbers of 100nM-C were not significantly lower than 100nMN-C and PBS-C (P>0.05) (Figure 9A).

2.5 Overexpressed hsa-miR-138-2-3p reduced survival fraction after radiation
100nM-TR, 100nM-CR, and non-transfection of Hep-2 cell line were treated with 0, 2, 4, 6, 8 Gy X-ray irradiation respectively, and then were incubated for additional 14 days. After incubation, cells were fixed with paraformaldehyde and the colonies (> 50 cells) were counted for each culture dish by a light microscope. The survival fraction (SF) of 100nM-TR, 100nM-CR, and non-transfection of Hep-2 CSCs were evaluated. The SF of 100nM-TR of all laryngeal CSCs were lower than that of 100nMN-CR and non-transfection, and the difference between 100nM-TR and non-transfection were statistically significant.

**Figure 10 Overexpressed hsa-miR-138-2-3p reduced survival fraction after radiation**

Figure 10A, 10B and 10C showed the comparison of survival analysis among 100nM-TR, 100nMN-CR and non-transfection of Hep-2, M2e, and TU212 cell lines after radiation respectively. The vertical and horizontal axis in the Figure 10 stood for survival fraction and does(Gy) respectively. 100nM-TR, 100nM-CR, and non-transfection of Hep-2, M2e and TU212 were treated with 0, 2, 4, 6, 8 Gy X-ray irradiation respectively, and the survival fraction of 100nM-TR of all laryngeal CSCs were lower than that of 100nMN-CR and non-transfection, and the difference between 100nM-TR and non-transfection were statistically significant (* P<0.05).

2.6 Overexpressed hsa-miR-138-2-3p promoted DNA damage after radiation

The degree of DNA damage after radiation was measured by comet assay. As shown in Figure 11, the appearance of “comet” with fragmented DNA (tail) being separated from undamaged nuclear DNA (head) was seen in 100nM-TR and 100nMN-CR of Hep-2, M2e, and TU212 CSCs after radiation. We found that the “heads” of “comet” of 100nM-TR were smaller than that of 100nMN-CR, while the “tails” of “comet” of 100nM-TR were longer than that of 100nMN-CR. Further, the measurements were made by Comet Assay IV software to calculate the tail movement (%). As Figure 12 shown, the tail movement of 100nM-TR of all laryngeal CSCs were higher than that of 100nMN-
CR, the differences between 100nM-TR and 100nMN-CR of Hep-2 cells were statistically significant. These data were indicated that the DNA damage of 100nM-TR were more serious than that of 100nM-CR in laryngeal CSCs after radiation.

Figure 11 Overexpressed hsa-miR-138-2-3p promoted DNA damage after radiation by Comet assay

Figure 11A and 11D showed the DNA damage analysis of 100nM-TR and 100nMN-CR of Hep-2 cell line after radiation respectively; Figure 11B and 11E showed the DNA damage analysis of 100nM-TR and 100nMN-CR of M2e cell line after radiation respectively; Figure 11C and 11F showed the DNA damage analysis of 100nM-TR and 100nMN-CR of TU212 cell line after radiation respectively. As shown in Figure 11, the appearance of "comet" with fragmented DNA (tail) being separated from undamaged nuclear DNA (head) was seen in 100nM-TR and 100nMN-CR of Hep-2, M2e, and TU212 CSCs after radiation. It was found that the "heads" of "comet" of 100nM-TR were smaller than that of 100nMN-CR, while the "tails" of "comet" of 100nM-TR were longer than that of 100nMN-CR. These data
were indicated that the DNA damage of 100nM-TR were more serious than that of 100nM-CR in laryngeal CSCs after radiation.

**Figure 12. Overexpressed hsa-miR-138-2-3p promoted DNA damage after radiation**

Figure 12A showed the comparison of DNA damage between 100nM-TR and 100nMN-CR of Hep-2 cell line after radiation respectively; Figure 12B showed the comparison of DNA damage between 100nM-TR and 100nMN-CR of M2e cell line after radiation respectively; Figure 12C showed the comparison of DNA damage between 100nM-TR and 100nMN-CR of TU212 cell line after radiation respectively. The vertical and horizontal axis in the Figure 12 standed for Tail movement (%) and treataments respectively. As Figure 12 shown, the tail movement of 100nM-TR of all laryngeal cell lines were higher than that of 100nMN-CR, and the differences between 100nM-TR and 100nMN-CR of Hep-2 CSCs were statistically significant (** P<0.01). These data were indicated that the DNA damage of 100nM-TR were more serious than that of 100nM-CR in laryngeal CSCs after radiation.

3. Overexpressed hsa-miR-138-2-3p regulated signal transduction pathway of laryngeal CSCs after radiation

3.1 Overexpressed hsa-miR-138-2-3p inhibited Wnt/β-catenin pathway

It was known that accumulation of β-catenin leaded to abnormal activation of Wnt/β-catenin signaling pathway and reinforce radiation resistance. As shown in Figure 13-14, the expression of β-catenin in
100nM-TR of all laryngeal CSCs were reduced more than that in 100nMN-CR, and the results between 100nM-TR and 100nMN-CR of all laryngeal CSCs were significant difference. It suggested that overexpression of hsa-miR-138-2-3p reduced the expression of β-catenin, further inhibited the activity of Wnt/β-catenin pathway and reduced the resistance of the laryngeal CSCs to radiation.

**Figure 13. Overexpressed hsa-miR-138-2-3p down-regulated expression of β-catenin**

As shown in Figure 13, the expression of β-catenin in 100nM-TR of Hep-2 (A), M2e (B) and TU212 (C) CSCs were reduced more than that in 100nMN-CR.

**Figure 14. Overexpressed hsa-miR-138-2-3p inhibited Wnt/β-catenin pathway**

As Figure 14 shown, the vertical and horizontal axis standed for Gary value and treaments respectively. It suggested that the expression of β-catenin between 100nM-TR and 100nMN-CR of Hep-2 (A), M2e (B) and TU212 (C) CSCs were significant difference (*** P<0.001).

### 3.2 Overexpressed hsa-miR-138-2-3p inhibited Hippo/YAP1 pathway

Hippo signal pathway regulated cell prolifertion, development and progression. Ancillary transcription factor YAP1 played a key role in Hippo pathway. Knocking out nuclear YAP1 can inhibit tumor cell proliferation, while increased expression of YAP1 can promote cell growth and migration and inhibit apoptosis. As shown in Figure 15-16, the expression of YAP1 in 100nM-TR of all laryngeal CSCs
were down-regulated much more than that in 100nMN-CR, and the results between 100nM-TR and 100nMN-CR of all laryngeal CSCs were significant difference. It suggested that overexpression of hsa-miR-138-2-3p reduced the expression of YAP1, further controlling Hippo signal pathway to weaken radio-resistance of laryngeal CSCs.

![Figure 15. Overexpressed hsa-miR-138-2-3p down-regulated expression of YAP1](image)

**Figure 15. Overexpressed hsa-miR-138-2-3p down-regulated expression of YAP1**

As shown in Figure 15, the expression of YAP1 in 100nM-TR of Hep-2 (A), M2e (B) and TU212 (C) CSCs were reduced more than that in 100nMN-CR.

![Figure 16. Overexpressed hsa-miR-138-2-3p inhibited Hippo/YAP1 pathway](image)

**Figure 16. Overexpressed hsa-miR-138-2-3p inhibited Hippo/YAP1 pathway**

As Figure 16 shown, the vertical and horizontal axis stooded for Gary value and treatments respectively. It suggested that the expression of YAP1 between 100nM-TR and 100nMN-CR of Hep-2 (A), M2e (B) and TU212 (C) CSCs were significant difference (**P<0.001**).

### 3.3 Overexpressed hsa-miR-138-2-3p activated JNK1/p38/MAPK pathway

MAPK/JNK signal pathway was activated by radiation, and enhanced the radio-sensitivity of tumor cells by its association with radiation-induced DNA damage [16,18]. As shown in Figure 17-19, the expression of JNK1 and p38 in 100nM-TR of all laryngeal CSCs were improved much more than that in 100nMN-CR, and the results between 100nM-TR and 100nMN-CR of all laryngeal CSCs were
significant difference. It suggested that overexpression of hsa-miR-138-2-3p promoted the expression of JNK1 and p38, further activating JNK1/p38/MAPK signal pathway to increase radio-sensitivity of laryngeal CSCs.

![Figure 17. Overexpressed hsa-miR-138-2-3p up-regulated expression of JNK1 and p38](image)

As shown in Figure 17, the expression of JNK1 and p38 in 100nM-TR of Hep-2 (A), M2e (B) and TU212 (C) CSCs were improved more than that in 100nMN-CR.

![Figure 18. Overexpressed hsa-miR-138-2-3p activated JNK1/MAPK pathway](image)

As Figure 18 shown, the vertical and horizontal axis standed for Gary value and treatments respectively. It suggested that the expression of JNK1 between 100nM-TR and 100nMN-CR of Hep-2 (A), M2e (B) and TU212 (C) CSCs were significant difference (** P<0.01, *** P<0.001).
Figure 19. Overexpressed hsa-miR-138-2-3p activated p38/MAPK pathway

As Figure 19 shown, the vertical and horizontal axis standed for Gary value and treatments respectively. It suggested that the expression of p38 between 100nM-TR and 100nMN-CR of Hep-2 (A), M2e (B) and TU212 (C) CSCs were significant difference (*P<0.05, ** P<0.01).

Discussion

In recent years, a large number of studies have focused on miRNA and cancer radiosensitivity. miRNAs played important roles in cell proliferation, invasion, apoptosis and cell cycle arrest, and aggressive growth, development, and metastasis are another characteristics of radio-resistance[16,23]. Balca-Silva et al[19]found that over-expressed miR-34 enhanced the radio-sensitivity of non-small cell lung cancers. In gastric cancer, knocking out miR-221 and miR-222 could inhibit proliferation and invasion, increase radiosensitivity of gastric carcinoma cells[20]. So far, however, there is no report on the relationship between hsa-miR-138-2-3p and radiation sensitivity of laryngeal cancer. In our present study, overexpressed hsa-miR-138-2-3p played an key role in decreasing laryngeal CSCs proliferation and invasion; increasing the proportion of early and late apoptosis in laryngeal CSCs; raising G1 phase arrest; and down-regulating the proportion of S stage cells of cell cycle that were related to radio-resistance in laryngeal CSCs.

Wnt/β-catenin signal transduction pathway regulated many cellular processes such as cell proliferation, apoptosis and aggressiveness, of which, β-catenin is the major factor. Che et al [23]
investigated that Cox-2 inhibitor NS398 inhibited the expression of DNA-PKCs and controlled Wnt/β-catenin pathway to improve the radiation sensitivity of Eca109 cells. Chang et al.[24] suggested that the radiation resistance of AMC-HN-9 cells were decreased remarkably when β-catenin was knocked out. Wang’s study[25] showed that the excessive expression of IncRNA–p21 improve the radiosensitivity of cells by inhibiting Wnt/β-catenin signaling pathway. Regulating upstream signaling molecules of Wnt/β-catenin pathway and promoting degradation of β-catenin may become new anticancer treatments [26-28]. Recent studies have revealed that Hippo signaling pathway played a role in the growth of many types of cancer cells, and YAP1 was associated with lung cancer and ovarian malignant tumors. Cell proliferation was inhibited by knocking out YAP1, while increased expression of YAP1 accelerated cell growth and migration. YAP1 was also called oncoprotein. MAPK signal pathway plays a role in regulating many cellular activities, such as growth, differentiation and stress reaction. Many signal transduction pathways related to radiation were regulated by MAPK family, including extracellular signal regulating kinase (ERK), c-Jun amino end kinase (JNK) and p38 MAPK pathway. JNK1 and JNK2, the stress inducing protein kinases, induce the phosphorylation of transcription factor c-Jun. MAPK pathway activated in this way is also called stress activating protein kinase (SAPK) pathway. Studies have shown that apoptosis initiation and cell cycle arrest were closely related to MAPK/p38 and MAPK/JNK1 pathway. Mediating MAPK/p38 and MAPK/JNK1 pathways can affect the radio-sensitivity of tumor cells. MAPK signaling pathway is considered to have an important effect on the radiation sensitivity of tumor cells for its association with radiation-induced DNA damage [16]. The activation of JNK and associated signaling pathway is related to apoptosis. Activation of SAPK/JNK leads to cell radio-sensitivity, and vice versa [18]. Wang et al.[28] and Bulavin et al.[29] found that MAPK/p38 signaling pathway regulated the transition of G2/M phase in mammalian cells and participated in G2 arrest. Wakita et al.[30] showed that Reg Iα-expressing cells activated MAPK/JNK1 pathway to increase JNK1 protein expression so as to improve the radio-sensitivity of esophageal squamous cell carcinomas. From the present study, we investigated that overexpression of hsa-miR-138-2-3p reduced the expressions of β-catenin and YAP1 in the laryngeal
CSCs after radiation, further Wnt/β-catenin and Hippo/YAP1 signal pathways were inhibited to weaken radio-resistance of laryngeal CSCs.

Conclusion
The present research indicated that overexpressed hsa-miR-138-2-3p played an key role in decreasing laryngeal CSCs proliferation and invasion; increasing the proportion of early and late apoptosis in laryngeal CSCs; raising G1 phase arrest; and down-regulating the proportion of S stage cells of cell cycle that were related to radio-resistance in laryngeal CSCs. Overexpressed hsa-miR-138-2-3p regulated signal transduction pathway of laryngeal CSCs after radiation. Over-expression of hsa-miR-138-2-3p down-regulated the expression of β-catenin and YAP1 in the laryngeal CSCs after radiation, further Wnt/β-catenin and Hippo/YAP1 signal pathways were inhibited to weaken radio-resistance of laryngeal CSCs. While the expressions of JNK1 and p38 were promoted, JNK1/p38/MAPK signal pathway was activated to increase radio-sensitivity of laryngeal CSCs. These results are useful for a better understanding of hsa-miR-138-2-3p in laryngeal CSCs, and prove hsa-miR-138-2-3p as a promising biomarker and target for diagnosis and novel anti-cancer therapy for laryngeal cancers.

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