MicroRNA-207 enhances radiation-induced apoptosis by directly targeting akt3 in cochlea hair cells

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MicroRNAs (miRNAs) have important roles in various types of cellular biological processes. Our study aimed to determine whether miRNAs function in the regulation of ionizing radiation (IR)-induced cell death in auditory cells and to determine how they affect the cellular response to IR. Microarray and qRT-PCR were performed to identify and confirm the differential expression of miRNAs in the cochlea hair cell line HEI-OC1 and in vivo after IR. Upregulation or downregulation of miRNAs using miRNA mimics or inhibitor were detected to characterize the biological effects of the indicated miRNAs. Bioinformatic analyses, luciferase reporter assays and mRNA knockdown were performed to identify a miRNA target gene. We determined that miR-207 was significantly upregulated after IR. MiR-207 enhances IR-induced apoptosis and DNA damage in HEI-OC1 cells. Furthermore, Akt3 was confirmed to be a direct target of miR-207. Downregulation of Akt3 mimics the effects of miR-207. MiR-207 enhances IR-induced apoptosis by directly targeting Akt3 and anti-miR-207 may have a potential role in protecting cochlea hair cells from IR.

Cell Death and Disease (2014) 5, e1433; doi:10.1038/cddis.2014.407; published online 2 October 2014

Radiotherapy (RT) is one of the most important treatments for head and neck (HN) cancers. Although the technologies for RT have greatly improved in recent years, the incidence of side effects induced by RT remains high. Sensorineural hearing loss (SNHL) is considered to be a principal complication of RT for HN and markedly affects the quality of life for patients with HN cancers.1 It has been demonstrated that the death of cochlea hair cells is responsible for ionizing radiation (IR)-induced SNHL.2–6 Regulators, such as p53, reactive oxygen species and c-Jun N-terminal kinases are known to have important roles in apoptosis of irradiated hair cells.7–10 N-acetylcysteine,11 epicatechin10 and metformin12 have been previously demonstrated to be effective in reducing apoptosis in irradiated hair cells. Because studies have been limited, the mechanisms underlying IR-induced auditory cell death remain unclear and require further investigation.

MicroRNAs (miRNAs) are a class of short noncoding RNAs. Some miRNAs have been extensively investigated. These RNAs repress translation or the stability of target mRNAs by binding to the 3’-untranslated regions (UTRs) of the mRNAs with imperfect complementarity.13,14 By regulating gene expression at the post-transcriptional level, miRNAs have important roles in various types of cellular biological processes, including responses to IR. However, most published studies on miRNAs regulating radiosensitivity have examined cancer cells, and little attention has been devoted to normal cells. Moreover, the relationship between miRNAs and IR-induced cochlea hair cell death has not been investigated to date.

In this study, we aimed to investigate IR-responsive miRNAs by analyzing the miRNA expression profile in the auditory cell line HEI-OC1 and identified miR-207 as an IR-inducible miRNA. MiR-207 enhanced apoptosis by increasing DNA damage in irradiated HEI-OC1 cells. Further investigation revealed that miR-207 negatively regulated Akt3 as a direct target. Thus, we provide a new mechanism for IR-induced apoptosis in cochlea hair cells. Taken together, our findings may help to develop a potential protectant for IR-induced SNHL.

Results

MiR-207 expression is induced by IR and inhibits cell growth. A differential miRNA expression profile between irradiated and nonirradiated HEI-OC1 cells was determined using microarray. These results revealed that three miRNAs were upregulated, and nine miRNAs were downregulated after IR when taking into account a fold-change > 6.3 (log2) and P < 0.05 (Table 1). We focused on the three upregulated miRNAs, including miR-207, miR-29c and miR-466i-5p, for further investigation. The qRT-PCR results confirmed that all these miRNAs were significantly upregulated at 12, 24 and 48 h after IR and the high expression remained stable (Figure 1a). After we successfully transfected miR-207, miR-29c and miR-466i-5p mimics individually into HEI-OC1 cells (Figure 1b), an methyl thiazolyl tetrazolium (MTT) assay was performed. Upregulation of miR-207 significantly inhibited cell growth in cells after IR (10, 20 Gy) but not in cells without IR, whereas miR-29c or miR-466i-5p overexpression did not exhibit any growth effect in irradiated or nonirradiated cells (Figure 1c). To confirm the expression of miR-207 in vivo, we performed assays on irradiated and control cochleas. Results from qRT-PCR, northern blotting and in situ hybridization (ISH) were identical and further verified the...
upregulation of miR-207 in irradiated cochleas (Figures 1d–f). On the basis of this finding, further studies were performed to determine how miR-207 affects cell growth.

**MiR-207 enhances IR-induced apoptosis.** The flow cytometry results for cell cycle analysis showed that populations of G1, S and G2 phases were not significantly different between miR-207 transfected and control cells after IR (Figure 2a), which indicated that miR-207 did not affect the distribution of cell cycle in irradiated cells. Next, we investigated whether miR-207 affected apoptosis in HEI-OC1 cells. The flow cytometry results for apoptosis indicated an upregulation of miR-207 significantly enhanced apoptosis compared with control in irradiated cells, whereas inhibition of miR-207 significantly mitigated apoptosis (Figure 2b). In cells without IR, no differences were found between groups treated with miR-207, miR-207 inhibitor or control. To confirm the apoptosis-enhancement effect of miR-207, western blotting analyses were performed. MiR-207 moderately increased the expression of cleaved PARP after IR, whereas inhibition of miR-207 greatly repressed cleaved PARP expression (Figure 2c). Furthermore, in cells treated without IR, the level of miR-207 did not affect the expression of cleaved PARP. On the basis of these studies, we concluded that miR-207 enhanced apoptosis, which only occurred in cells with IR.

**MiR-207 enhances IR-induced DNA damage.** Next, we investigated whether increased apoptosis by miR-207 is

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**Table 1** Differential miRNAs expression in HEI-OC1 cells after irradiation

| miRNA ID  | lg (ratio) | P-value  |
|-----------|------------|---------|
| miR-207   | 1.13       | 3.73E-05|
| miR-29c   | 0.96       | 4.96E-03|
| miR-466i-5p | 3.00   | 4.51E-05|
| miR-101a-5p | -1.04 | 1.03E-05|
| miR-1247-5p | -1.19 | 2.12E-05|
| miR-1899  | -1.12      | 4.99E-06|
| miR-222   | -1.22      | 1.80E-04|
| miR-3473d | -1.37      | 6.99E-05|
| miR-375-5p | -1.24 | 8.82E-06|
| miR-491-5p | -1.22 | 1.74E-04|
| miR-5100  | -1.09      | 7.67E-04|
| miR-719   | -1.11      | 2.41E-04|

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**Figure 1** miR-207 expression is induced by IR and inhibits cell growth. (a) qRT-PCR was performed to confirm the upregulated expression of miR-207, miR-29c and miR-466i-5p in HEI-OC1 cells at 12, 24 and 48 h after 20 Gy irradiation. U6 spliceosomal RNA was used for normalization. Error bar, S.D.; *P<0.05 (n=3) versus the nonirradiated (0 h) group, **P<0.001 (n=3) versus the nonirradiated (0 h) group. (b) HEI-OC1 cells were transfected with miR-207, miR-29c and miR-466i-5p independently. qRT-PCR was performed to detect the level of the indicated miRNAs at 48 h after transfection. Error bar, S.D.; *P<0.01 (n=3) versus control. (c) HEI-OC1 cells transfected with miR-207, miR-29c, miR-466i-5p and control miRNAs were subjected to the MTT assay at 24 h after IR (10, 20 Gy) or without IR. Error bar, S.D.; *P<0.001 (n=3) versus control. (d) qRT-PCR, (e) northern blotting and (f) *in situ* hybridization were performed to confirm the upregulated expression of miR-207 in cochlea at 24 h after 20 Gy irradiation on mice. U6 was used for normalization. Error bar, S.D.; *P<0.001 (n=5) versus control. OHC = outer hair cell.
associated with an enhancement in DNA damage. Transfection with miR-207 resulted in higher γ-H2AX expression at 6 and 12 h after IR compared with control. In contrast, transfection with miR-207 inhibitors resulted in significantly lower γ-H2AX expression independent of time (6 or 12 h) after IR (Figure 3a). We also assessed the DNA damage by quantifying γ-H2AX foci after staining. HEI-OC1 cells with upregulated miR-207 expression showed significantly more foci in the nucleus, whereas cells with downregulated miR-207 expression showed the opposite result. Taken together, these results suggest that miR-207 significantly enhanced IR-induced DNA damage.

**Akt3 is a direct target of miR-207.** To identify the target mRNA of miR-207, we used five miRNA target prediction programs, including EIMMo, miRanda, miTarget, PicTar and TargetScan. As shown in Figure 4a, the complementary sequence for the seed region of miR-207 was at position 1184-1191 of Akt3 3'-UTR. A reporter (WT 3'-UTR) containing the exact complementary sequence in the Akt3 3'-UTR fragment and another reporter (Mut 3'-UTR) containing mutated nucleotides of the complementary sequence were constructed (Figure 4a). Expectedly, co-transfection with miR-207 and WT 3'-UTR reporter significantly reduced luciferase activity compared with control (Figure 4b). In addition, Akt3 mRNA and protein levels significantly decreased after miR-207 transfection (Figures 4c and d). However, the expression of other Akt isoforms, including Akt1 and Akt2, remained unchanged (Figure 4d). Moreover, P-Akt increased markedly after IR, but decreased moderately in cells transfected with miR-207. Taken together, these results revealed that Akt3 is a direct target of miR-207.

**Downregulation of Akt3 mimics the effects of miR-207.** We next examined whether downregulation of Akt3 exhibited similar effects of miR-207. First, we specifically
repressed the expression of Akt3 protein, but not Akt1 or Akt2 (Figure 5a). Apoptosis analysis showed that HEI-OC1 cells transfected with miR-207 or siAkt3 exhibited significantly greater apoptosis compared with control (Figure 5b). At the protein level, western blotting analyses detected the expression of γ-H2AX as well as cleaved PARP, which increased similarly between cells transfected with miR-207 and siAkt3 (Figure 5c).

**Discussion**

MiRNAs are indispensible for cochlea hair cell maintenance and survival.\(^\text{16}\) The expression of miRNA changes while the cochlea is under various stress, eventually causing cell death and SNHL.\(^\text{17}\) Oxidative stress upregulates 35 miRNAs and downregulates 40 miRNAs in HEI-OC1 cells.\(^\text{18}\) The miR-183/\(Taok1\) target pair and miR-34 family are found implicated in cochlear responses to acoustic trauma and kanamycin ototoxicity, respectively.\(^\text{19,20}\) In our study, miR-207, miR-29c and miR-466i-5p were identified as upregulated miRNAs in HEI-OC1 cells after IR, and miR-207 was confirmed to be the only one that affects cell viability. These evidences show that different stress may cause different miRNA expression in cochlea cells, which is probably because different miRNAs take part in different cellular processes. To the best of our knowledge, miR-207 has not been thoroughly investigated. MiR-207 was found to be downregulated in liver tissue after partial hepatectomy in mice\(^\text{21}\) and upregulated in a neuronal...
cell line (MN9D) with 6-hydroxydopamine (6-OHDA) treat-
ment, a component of a neurotoxin. Although these studies
demonstrated changes in miR-207 expression, they did not
investigate the function of miR-207. Our study reveals the
biological function of miR-207 and proposes a miRNA
correlated to IR-induced injury in auditory cells.

Further studies have revealed that inhibition of cell growth
by miR-207 is caused by increased cell apoptosis rather than
cell cycle arrest. Moreover, an enhancement of apoptosis by
miR-207 was only observed in irradiated cells, which
suggested that this change is associated with IR-induced
DSBs. It is known that DNA is the major target of radiation
effects. The unsuccessful repair of DSBs may result in lethal
consequences, such as apoptosis, for cells. Moreover, γ-H2AX is one of the earliest markers of DSBs after IR. In the
present study, the expression of γ-H2AX was greatly
increased and was sustained in cells upregulated with
miR-207. This finding indicated that miR-207 enhanced IR-
induced DNA damage, which results in enhanced apoptosis.

Akt3 has been shown to be a direct target of miR-207 in our
study. Isoforms of the Akt family, including Akt3, Akt1 and Akt2,
share a high degree of structural similarity, but express and
function differently in specific cell types and biological
processes. Akt3 has been studied extensively in cancer cells
for its effects on cancer development, proliferation and
migration, however, little is known regarding its function in
normal cells. In our study, Akt3 was found to be specifically
inhibited by miR-207. Inhibition by siAkt3 resulted in an
enhancement of apoptosis and DSBs in irradiated HEI-OC1
cells, similar to results obtained with miR-207 treatment. This
indicated Akt3 potentially has an important role in miR-207-
mediated IR response in auditory cells. In addition, Akt1 and
Akt2 were shown to be important regulators in the activation of
DNA-dependent protein kinase, a key enzyme of nonhomo-
logous end joining of the DNA repair pathway. Akt3 may
share a DSB repair pathway with Akt1 and Akt2, and further
studies are required.

As previously mentioned, the therapeutic options against
IR-induced cochlea hair cell death are notably limited. Thus,
the identification of new therapeutic agents is important.
Although we have identified many genes related to apoptosis
induced by IR, it is not easy to directly manipulate these genes.
Among the regulators of protein-coding genes, miRNA is an
ideal choice. In our study, specific inhibition of miR-207
exhibits the exciting potential to protect HEI-OC1 cells from IR
by reducing apoptosis and DSBs. Thus, further studies are
required to confirm the protective effect of anti-miR-207 in vivo.
Conclusions

In summary, our study is the first to indicate that overexpression of miR-207 enhances apoptosis in irradiated auditory cells. Moreover, the enhancement of apoptosis is potentially caused by the disruption in DSBs repair. We also determined that specific inhibition of miR-207 significantly mitigated damages induced by IR on HEI-OC1 cells, which may represent a novel strategy in protecting IR-induced SNHL. Furthermore, we confirmed Akt3 is a direct target for miR-207. Thus, additional studies are needed to clearly reveal the molecular mechanisms underlying the role of Akt3 in miR-207-enhanced apoptosis after IR.

Materials and Methods

Cell culture. The HEI-OC1 cell line was generously provided by F. Kalinec (House Ear Institute, Los Angeles, CA, USA). It is a conditionally immortalized organ of the Corti-derived epithelial cell line, which has been used to investigate the cellular and molecular mechanisms of ototoxicity induced by drugs, noise or irradiation. This cell line was maintained in high-glucose Dulbecco’s modified Eagle’s medium (Gibco, Cergy-Pontoise, France) containing 10% fetal bovine serum (Gibco) without antibiotics at 33°C under 10% CO2 in an incubator.

Animals. C57BL/6 mice were purchased from the Southern Medical University Laboratory. Animal care and killing were conducted according to methods approved by the Southern Medical University Animal Care and Use Committee, following guidelines of National Institute of Health for use of laboratory animals. 10 C57BL/6 mice were divided into two groups (control group and irradiation group).

Irradiation. The HEI-OC1 cells were irradiated at a distance of 100 cm from the source to the axis using a 6-MV linear accelerator (LINAC; 2300EX; Varian Co., Palo Alto, CA, USA) at a dose rate of 5.0 Gy/min. The mice of radiation group were placed and fixed in the prone position on a plate after they were anaesthetized with 40 mg/kg 0.1% pentobarbital sodium. The mice then received irradiation restricted to the head. A single dose of 20 Gy was delivered by opposed beams bilaterally with a distance of 100 cm from the source to the axis.

miRNA microarray. The expression of miRNAs in IR-treated versus untreated cells was analyzed using miRNA microarray. The total RNA was extracted from the untreated cells or cells 24 h post IR (20 Gy) using Trizol (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the manufacturer. Determination of the quantity and quality of the extracted RNA and the microarray experiment were performed by Guangzhou RiboBio Co. Ltd. using Mouse & Rat miRNA OneArray V3.0 (Phalanx Biotech Company, Hsinchu, Taiwan).

qRT-PCR. After isolation from the IR-treated or untreated cells or cochlea, the total RNA was reverse-transcribed into cDNA using the PrimeScript RT reagent kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. qPCR was performed using the ABI PRISM 7500 Fast Real-Time PCR System (Perkin Elmer/ Applied Biosystems, Rotkreuz, Switzerland) with a SYBR Premix Ex Taq II kit (TaKaRa). The small noncoding RNA U6 and housekeeping gene GADPH were used as calibrators for data analysis.
used as an internal control for miRNA and Akt3 quantification, respectively. The sequences of the gene-specific primers used for qPCR are shown as follows: miR-207: forward 5′-ACACTCCAGCGGCTTCTCGGTC-3′; reverse 5′-CCCTCCACCAAGGCGTTTATA-3′; miR-29c: forward 5′-ACACTCCAGGTGGTAGCACATTGTT-3′; miR-466i-5p: forward 5′-ACACTCCAGGTGGTGTTGGTGT-3′; universal reverse primer for miRNAs: 5′-TGTTGCTGGAGTCG-3′; Akt3: forward 5′-ACGGCACACGGTTC-3′; reverse 5′-CCCTCCACCAAGGCGTTTATA-3′; U6: forward 5′-CTCGTTGCGGAGAACATTGG-3′; GADPH: forward 5′-GTTGGCTGGGGCTCTACTTC-3′; reverse 5′-GTGGCTGGGGCTCTACTTC-3′. All reactions were performed in triplicate for each sample.

Northern blotting. The locked nucleic acid (LNA) probe for miR-207 labeled with digoxigenin (DIG Oligonucleotide 3′-End LABELING KIT; Roche, Stockholm, Sweden) were purchased from Exiqon Co (Vedbaek, Denmark). U6 RNA was detected using a DIG-labeled U6 DNA probe. Northern blotting was performed as described.31 Total RNA from cochleas was resolved by 15% denaturing gel. We estimated the DNA damage by performing immunofluorescent staining for H2AX primary antibody (1: 100 dilution; Abcam, San Francisco, CA, USA). Next, the cells were washed in PBS, and rabbit anti-mouse AlexaFlour-488 secondary antibody (1: 200 dilution; Abcam) was applied for 1 h at room temperature in the dark. After three 5 min washes in PBS, the samples were mounted in fluorescence mounting medium with DAPI. The cells were observed using a fluorescence microscope (Olympus, Shinjuku-ku, Tokyo, Japan), and the γH2AX foci were manually quantified in at least three individual fields of ~100 cells.

Cell cycle analysis using flow cytometry. The transfected cells were collected at 24 h after IR (20 Gy) or without IR. Next, cells were washed with PBS once and resuspended in PBS containing 0.2% Triton X-100 for permeabilization and propidium iodide (PI) staining. After incubation for 30 min, the cells were analyzed using flow cytometry. Each experiment was performed in triplicate.

Apoptosis analysis using flow cytometry. The transfected cells were collected at 24 h post IR (20 Gy) or without IR treatment. Cells undergoing apoptosis were determined by staining with Alexa Fluor 488 annexin V and PI according to the protocol provided by the manufacturer (Invitrogen). Each experiment was performed in triplicate.

Western blotting. Cells were collected and western blot analyses were performed as previously described.25 β-actin was used as the loading control. The primary antibodies used for western blotting included: rabbit anti-cleaved PARP (9544, Cell Signaling, Beverly, MA, USA), anti-H2AX (#9718, Cell Signaling), anti-Akt1 (#9514, SAB), anti-Akt2 (#8751, SAB), anti-Akt3 (#4059, Cell Signaling), anti-pAkt (#4690, Cell Signaling) and anti-β-actin (#4970, Cell Signaling).

Bioinformatic analyses. We obtained the mature sequence of miR-207 from the miRNA database (http://www.mirbase.org/). EIMMo (http://www.mir.unicas.it/EIMMo2/), miRanda (http://www.micromat.de/), miTarget (http://cbit.snu.ac.kr/~mtarget/), PicTar (http://pcTar.molbio.dtu.dk/) and TargetScan (http://www.targetscan.org/) was used to predict the target gene of miR-207. The 3′-UTR of Akt3 gene containing the predicted target sites of miR-207 was amplified by PCR using the primers 5′-TACAGATCATGGGCCGCTG-3′ for Akt3-3′-UTR-forward and 5′-TCCACACCTCGGCTTCAC-3′ for Akt3-3′-UTR-reverse. The amplified sites were cloned into the pIRE2-m-cherry vector (Clontech, Madison, WI, USA). A mutant 3′-UTR of Akt3 with a mutated sequence (5′-AAUCUGUCUCUCUGAGCUGUGA…-3′), the mutated sites are underlined in the complementary site for the miR-207 seed region was also amplified and cloned into the PLUC-REPORT vector. For the reporter luciferase assay, HEI-O1 cells were cultured in 96-well plates and co-transfected with pLUC-3′-UTR-Akt3 (Akt3-WT) or pLUC-3′-UTR-Mut-Akt3 (Akt3-Mut) and miR-207 mimics or control with Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). Forty-eight hours after transfection, cells were assayed for luciferase activity with the Dual-Luciferase reporter assay kit (Promega). Experiments were performed in triplicate.

Statistical analyses. Data were analyzed using the Student’s t-test or one-way ANOVA for statistical significance. Statistical evaluations are presented as the mean ± S.D., and a P-value of < 0.05 was considered to be statistically significant.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements. We acknowledge the excellent technical support and sincere help of the Functional Genomics and Bioinformatics cores of Southern Medical University. We also thank Prof. F Kalinec (House Ear Institute, Los Angeles, CA, USA) for kindly providing the HEI-O1 cells. This study was supported by the National Natural Science Foundation of China (81272508, 81302385), Specialized Research Fund for the Doctoral Program of Higher Education of China (2011443110015), Guangzhou Science and Technology Project Fund (11C22120714) and Guangdong Provincial Department of education research project (2013JKX0038).

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