miR-608 regulates apoptosis in human lung adenocarcinoma via regulation of *AKT2*

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Abstract. Lung cancer remains a major health problem with a low 5-year survival rate of patients. Recent studies have shown that dysregulation of microRNAs (miRNAs) are prevalent in lung cancer and these aberrations play a significant role in the progression of tumour progression. In the present study, bioinformatics analyses was employed to predict potential miR-608 targets, which are associated with signaling pathways involved in cancer. Luciferase reporter assay identified *AKT2* as a novel target of miR-608, and suppression of its protein levels was validated through western blot analysis. Zebrafish embryos were microinjected with cells transfected with miR-608 to elucidate the role of miR-608 *in vivo*, and immunostained with antibodies to detect activated caspase-3. We present the first evidence that miR-608 behaves as a tumour suppressor in A549 and SK-LU-1 cells through the regulation of *AKT2*, suggesting that selective targeting of *AKT2* via miR-608 may be developed as a potential therapeutic strategy for miRNA-based non-small cell lung cancer (NSCLC) therapy.

Introduction

Cancer is the leading cause of death worldwide, with lung cancer being the most common type of cancer, with an estimated 1.8 million new cases in 2012 (1). Even with administration of treatment, the 5-year survival rate for lung cancer is very low (17.7%) in comparison to other leading cancer sites such as colon (64.4%), breast (89.7%) and prostate (98.9%) (2). While control for early stage localized lung cancer has improved (3,4), early stage diagnosis only accounts for ~16% of lung cancer (2), with majority of patients being diagnosed at an advanced or metastatic stage of disease. Thus, it is of grave importance to further understand the molecular mechanisms regulating lung carcinogenesis and to explore and identify novel diagnostic biomarkers for treatment strategies.

MicroRNAs (miRNAs) are a subset of non-coding RNAs of ~19-23 nucleotides in length, which post-transcriptionally regulate gene expression (5). miRNAs play a role in crucial biological processes including proliferation (6,7), differentiation (8), chemosensitivity (9,10) and apoptosis (11,12). Studies have shown that aberrations in the expression of certain miRNAs may cause or contribute to human diseases, including cancer (13). Evasion of apoptosis is a major contributor to tumour progression, and past studies have elucidated that manipulation of the apoptotic process is one way by which miRNAs influence the development of lung cancer (11,14-17).

miR-608 is a novel prognostic marker in carcinogenesis, its expression is dysregulated in various cancers (18-21). A previous study by our group demonstrated that downregulation of B-cell lymphocyte xL (*BCL-XL*), the other major prototype of the anti-apoptotic bcl-2 gene, dysregulates various miRNAs in lung adenocarcinoma cell line A549, including miR-608. The study further shows that ectopic expression of miR-608 was able to increase cell death in non-small cell lung cancer (NSCLC) cells, and co-transfection of siRNA targeting *BCL-XL* (si*BCL-XL*) followed by miR-608 inhibitors was able to block si*BCL-XL* induced cell death, suggesting that miR-608 plays an important role in cell death processes (22).

In the present study, we evaluated the role of miR-608 NSCLC and the molecular mechanisms by which it regulates apoptosis. Our data identified miR-608 as a tumour suppressor in NSCLC, through identification of a novel direct target responsible for mediating the activity of miR-608 in NSCLC.

Materials and methods

Bioinformatics analyses of miRNA gene targets. In silico analyses was performed to identify the putative miRNA targets using TargetScan Human V5.2 (http://www.targetscan.org/) (Whitehead Institute for Biomedical Research, Cambridge, MA, USA), a database of conserved 3'UTR targets. TargetScan provides accurate ranking of the predicted targets of miRNA based on total context+ score, which is the sum of the contribution of six targeting factors including site type, site number, site location, local AU content, 3'-supplementary pairing, target site abundance and seed-pairing stability. The total context+

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score predicts the relative repression of mRNA with 3'UTR, with low context scores being more favorable (23). The web tool Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/summary.jsp) (SAIC-Frederick, Inc., Frederick, MD, USA), which is made up of an integrated biological knowledgebase and analytic tools (24), was then employed, using default parameters, to perform gene-annotation enrichment analyses on TargetScan's predicted miRNA targets that has a total context+ score of <0. Data from TargetScan and DAVID were combined to generate a hypothetical pathway of the relationship between the miRNAs and their gene targets.

Cell lines and culture conditions. Human lung adenocarcinoma cell line A549 [Cancer Research Initiative Foundation (CARIF), Subang Jaya Medical Centre, Subang Jaya, Malaysia] was cultured in RPMI-1640 (SH30027.01; HyClone Laboratories-GE Healthcare Life Sciences, Pittsburgh, PA, USA) whereas SK-LU-1 cells (LA-HL-045; AseaCyte, Pvt. Ltd., Kuala Lumpur, Malaysia) were cultured in MEM-α (32561-037; Gibco, Waltham, MA, USA). All cells were supplemented with 10% fetal bovine serum (FBS) (SV30160.03; HyClone Laboratories-GE Healthcare Life Sciences) and maintained at 37˚C in a humidified incubator containing 5% CO_2.

miRNA transfection. Cells were seeded 24 h prior to transfection with miR-608 mimics (C-300933-01-0010; GE Healthcare Dharmacon, Lafayette, CO, USA), non-specific mimic controls (mimic NC) (CN-001000-01-20; GE Healthcare Dharmacon), miR-608 inhibitors (IH-300933-03-0010; GE Healthcare Dharmacon) or non-specific antimiR controls (inhibitor NC) (IN-001005-01-20; GE Healthcare Dharmacon) at a final concentration of 80.0 nM using DharmaFECT reagent (T-2001-03; GE Healthcare Dharmacon), as per the manufacturer's protocol.

Dual-luciferase reporter assay system. Wild-type 3'UTR of *AKT2* containing predicted miR-608 binding sites and/or its corresponding mutant sequences were cloned into the pmirGLO Dual-Luciferase miRNA expression vector (E1330; Promega, Madison, WI, USA). A549 cells were plated 24 h prior to co-transfection with 40.0 ng of pmirGLO constructs and 80.0 nM of miR-608 mimic/inhibitor or mimic NC/inhibitor NC using DharmaFECT reagent. Luciferase activity was analyzed 48 h post-transfection using the Dual-luciferase reporter assay system (E2920; Promega), as per the manufacturer's protocol and detected on the GloMax Multi Luminescence Multimode Reader (Promega). Relative luciferase activity was normalized against *Renilla* luciferase activity.

Protein extraction and western blotting. Protein was extracted using the NE-PER® Nuclear and Cytoplasmic Extraction kit (78833; Thermo Fisher Scientific, Waltham, MA, USA) 48 h post-transfection, as per the manufacturer's protocol. Protein lysates were separated by electrophoresis in 12% SDS-PAGE and then electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in 1X Tris-buffered saline (TBS) with 0.05% Tween-20 and 5% non-fat skim milk powder (115363; Merck, Kenilworth, NJ, USA) for 1 h at room temperature and then immunostained overnight at 4˚C with primary monoclonal rabbit antibodies: AKT (4691, 1:1,000 dilution; Cell Signaling Technology, Danvers, MA, USA) or GAPDH (2118, 1:10,000 dilution; Cell Signaling Technology). The following day membranes were washed and incubated with secondary goat anti-rabbit IgG HRP-linked antibody (7074, 1:1,000 dilution; Cell Signaling Technology) and anti-biotin HRP-linked antibody (7075, 1:1,000 dilution; Cell Signaling Technology). Bands were visualized using WesternBright Quantum (K-12042-D10; Advansta, Inc., Menlo Park, CA, USA) on the Fusion FX7 system (Vilber Lourmat GmbH, Eberhardzell, Germany) and quantified using the ImageJ Analyst software (National Institutes of Health, Bethesda, MD, USA), with band intensities normalized to GAPDH.

Annexin V-FITC apoptosis assay. FITC Annexin V apoptosis detection kit (556547; BD Biosciences, San Jose, CA, USA) was used to detect cell death 72 h post-transfection, as per the manufacturer's protocol. Signals were detected from $1.0x10^4$ cell population using the BD FACSCanto™ II flow cytometer (BD Biosciences) and examined on the BD FACSDiva™ software (BD Biosciences).

Caspase-3/7 activity assay. Caspase-Glo 3/7 assay kit (G8090; Promega) was utilized to analyze caspase-3 and -7 activity, 48 h post-transfection as per the manufacturer's protocol. Samples were incubated at 25˚C for 1 h in the dark and luminescence was then detected using the GloMax Multi Luminescence Multimode Reader.

Cell cycle analysis. Flow cytometry was used to analyze cell cycle using the BD Cycletest™ Plus DNA kit assay (340242; BD Biosciences) 48 h post-transfection, as per the manufacturer's protocol. Signals were detected from $1.0x10^4$ cell population using the BD FACSCanto™ II flow cytometer and examined on the BD FACSDiva™ software. Results were then analyzed using the ModFit LT v3.2.1 (Verity Software House, Inc., Topsham, ME, USA) and the percentage of the cells in G0/G1, S and G2/M phase were counted and compared.

Zebrafish care and use. Experiments involving zebrafish were approved by the University of Malaya, Faculty of Medicine, Institutional Care of Use Committee (FOM IACUC) (Ethics reference number: 2015-181006/IBS/R/NO) and complied with all relevant animal welfare laws, guidelines and policies. Wild-type *Danio rerio* zebrafish embryos were cared for and maintained using standard husbandry practices.

Zebrafish microinjection. Zebrafish embryos were injected with A549 cells transfected with 80.0 nm miR-608 mimics, inhibitors, or their corresponding negative controls at the superficial location of the yolk near to the perivitelline space of the embryos using a FemtoJet Microinjector (Eppendorf, Hamburg, Germany) and InjectMan NI 2 Micromanipulator (Eppendorf) with constant injection pressure and injection time. The injection volume and cell suspension was calibrated to be ~100-200 cells/injection in each embryo. After transplantation, embryos were immediately placed at 37˚C overnight.

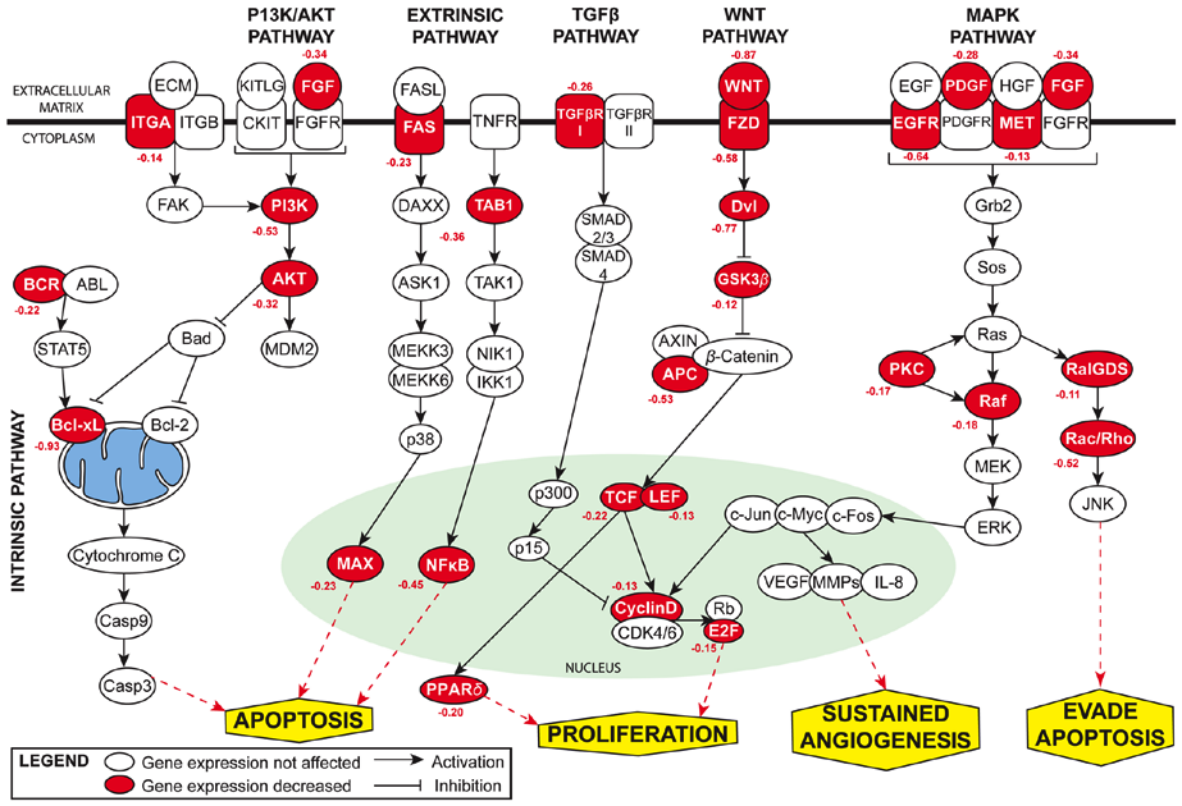

Figure 1. A hypothetical signaling network depicting the interactions of miR-608 and its putative targets. miR-608 is predicted to play a role in various biological processes including apoptosis, proliferation and angiogenesis. Numbers in red indicate total context score for that specific target with miR-608.

Whole mount caspase-3 immunofluorescence. Embryos were fixed in 4% paraformaldehyde at 4˚C overnight followed by 2-h dehydration in methanol at -20˚C. Following rehydration, embryos were washed with 1% dimethyl sulfoxide (DMSO), 0.1% Triton in PBS (1X PDT) and blocked with 10% FBS, 2% BSA in PBST (blocking buffer) for 1 h at room temperature. Embryos were then stained with purified rabbit anti-active caspase-3 antibody (559565, 1:500 dilution; BD Biosciences) for 2 h at room temperature followed by washes in PDT. Again embryos were incubated with blocking buffer, and then stained with anti-rabbit IgG Fab2 Alexa Fluor 647 Conjugate (4414, 1:500 dilution; Cell Signaling Technology) overnight at 4˚C. The following day, embryos were washed with PDT before visualization and imaging using the Leica confocal laser-scanning microscope SPII and Leica Application Suite (LAS) software v5.0 (Leica Microsystems, Wetzlar, Germany). Fluorescence was quantified using ImageJ Analyst software. Threshold was set to eliminate background fluorescence and embryos were analyzed to generate arbitrary fluorescence units.

siRNA silencing of AKT2. Silencing of the *AKT2* gene was performed using a set of three unique 27 mer siRNA duplexes at a final concentration of 10.0 nM (siRNA A: GCAUCAUA AAUUGGUAGUUUCCUGC, siRNA B: AGCGUGUGAAUA CAUCAAGACCTG, siRNA C: ACAGCAAAGCAGGAG UAUAAGAAAG) (SR300144; Origene Technologies, Inc., Rockville, MD, USA). A universal scrambled negative control siRNA (siRNA NC) was used as a control. At 48 h post-transfection, silencing efficiency was assessed by western blot analysis. Amongst the three siRNAs utilized, the siRNA with the greatest silencing efficiency was selected for further downstream work and referred to as si*AKT2*. The effects of miR-608 mediated apoptosis via *AKT2* was validated through transfection of 80.0 nM miR-608 inhibitors, followed by transfection with 10.0 nM si*AKT2* directed against the human AKT2 gene (si*AKT2*) 6 h later. AKT2 protein levels were determined via western blot analysis 48 h post-transfection while apoptosis was detected using the FITC Annexin V apoptosis detection kit and Caspase-Glo 3/7 assay kit.

Statistical analysis. All *in vitro* experiments were performed in triplicate independent experiments. *In vivo* experiments were performed with sample size of 15 zebrafish embryos per treatment group. All data were presented as mean ± standard deviation (25). Paired Student's t-test was used to determine the statistical significance of results, whereby a P-value of ≤0.05 was considered significant.

Results

miR-608 is predicted to bind to AKT2 3'UTR. A previous study conducted by our laboratory determined that the expression of miR-608 was significantly downregulated following the silencing of *BCL-XL* in lung adenocarcinoma cell line A549. Results also indicated that miR-608 played a tumour suppressor role in regulating the apoptotic properties of A549 and a secondary lung adenocarcinoma cell line SK-LU-1 (22). To determine the molecular mechanism by which miR-608 regulates the apoptotic properties in NSCLC cell lines, we

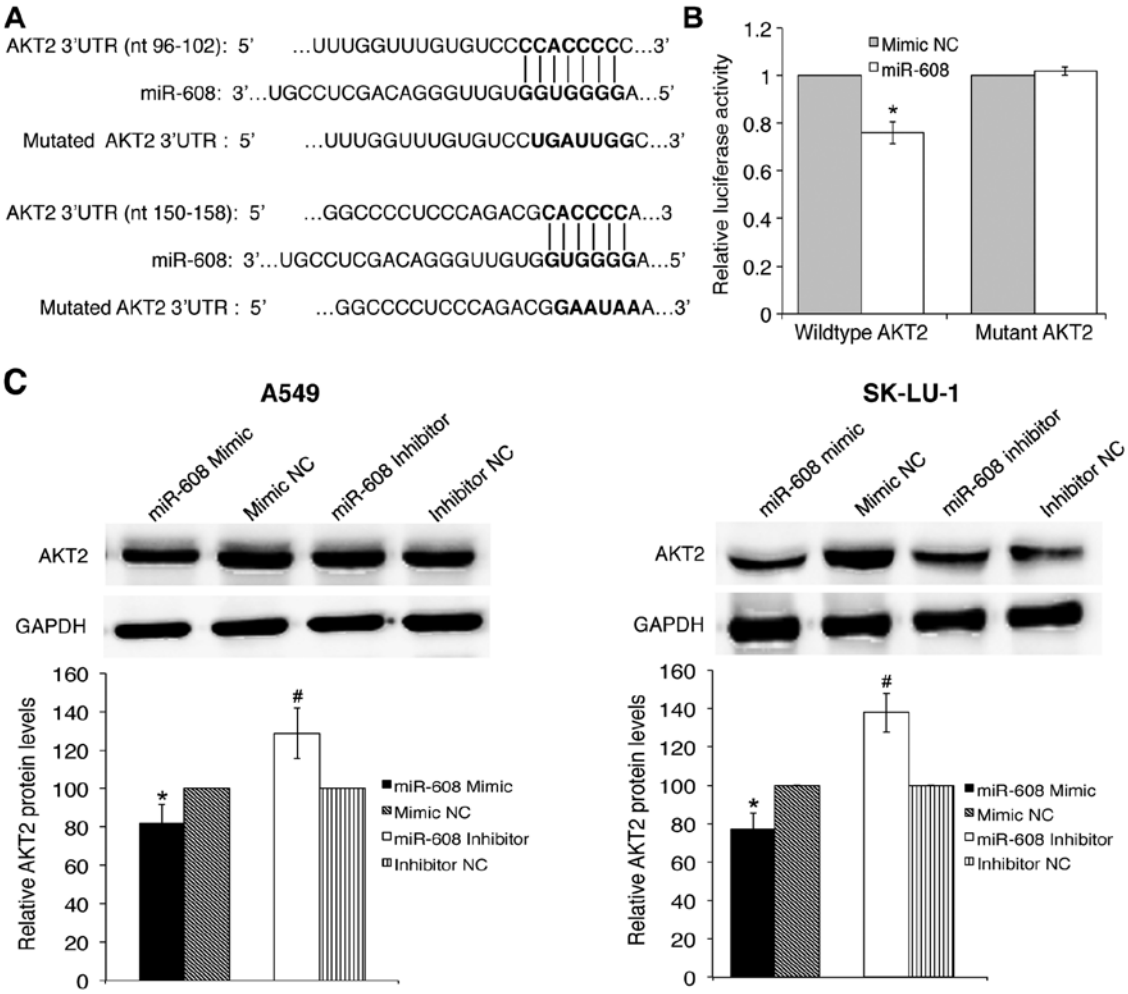

Figure 2. miR-608 directly targets *AKT2*. (A) Sequence alignment of miR-608 and *AKT2* 3'UTR. *AKT2* 3'UTR contains two predicted miR-608 binding sites at nucleotide 96-102 and 150-158. (B) Normalized relative luciferase activity in wild-type and mutant pmirGLO constructs in response to transfection with miR-608 mimic or mimic negative control in A549 cells. Samples were normalized to *Renilla* luciferase activity. (C) Relative AKT2 protein level following miR-608 mimic, mimic NC, miR-608 inhibitor and inhibitor NC transfection in A549 cells and SK-LU-1 cells. Statistically significant differences between mimic transfected groups and mimic NC groups are denoted with $^{*}P \leq 0.05$, while statistically significant differences between inhibitor transfected groups and inhibitor NC groups are denoted with $^{\#}P \leq 0.05$.

performed an *in silico* bioinformatics analysis to identify the putative miR-608 gene targets through the use of the TargetScan Human v5.2 algorithm, followed by functional annotation using the web tool DAVID v6.7, which lists the predicted targets of miR-608 according to their apoptosis-related pathways. miR-608 was found to be associated with various signaling pathways involved in cancer, including the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), wingless-type MMTV integration site family (WNT), transforming growth factor (TGF-β), mitogen activated protein kinase (MAPK) and the intrinsic and extrinsic pathway (Fig. 1).

Identification of AKT2 as a direct target of miR-608 in NSCLC cells. The 3'UTR of V-Akt Murine Thymoma Viral Oncogene Homolog 2 (*AKT2*) contains two miR-608 binding sites, and is involved with apoptosis and proliferation and was thus chosen for further validation. To verify whether *AKT2* 3'UTR was a direct target of miR-608, the wild-type and mutated *AKT2* 3'UTR were cloned into the pmirGLO Dual-luciferase miRNA target expression vector (Fig. 2A). Luciferase reporter assay confirmed that miR-608 mimics had a significant inhibitory effect on wild-type 3'UTR but not on the mutant 3'UTR of *AKT2* luciferase activity, while mimic NC had no effect on either the wild-type or mutant luciferase activity (Fig. 2B). This result suggests that miR-608 directly binds to the binding sequence of *AKT2* 3'UTR, and this was further verified by a decrease in AKT2 protein levels in response to miR-608 mimic transfection, as analyzed by western blot analysis. Conversely, the expression of AKT2 was significantly increased when miR-608 was inhibited (Fig. 2C).

siRNA-mediated silencing of AKT2 restores miR-608 induced effects in NSCLC cells. We have previously demonstrated that miR-608 plays an important role in the regulation of apoptosis, and presently identified miR-608 as a direct regulator of *AKT2*. It was thus hypothesized that low expression of miR-608 in NSCLC may result in suppression of its inhibitory effects towards *AKT2* causing AKT2 expression to be upregulated, which in turn blocks apoptosis. To investigate this hypothesis, co-transfection of miR-608 inhibitors and siRNA inhibiting *AKT2* was performed in A549 and SK-LU-1 cells. siRNAs were provided as a set of three siRNA duplexes;

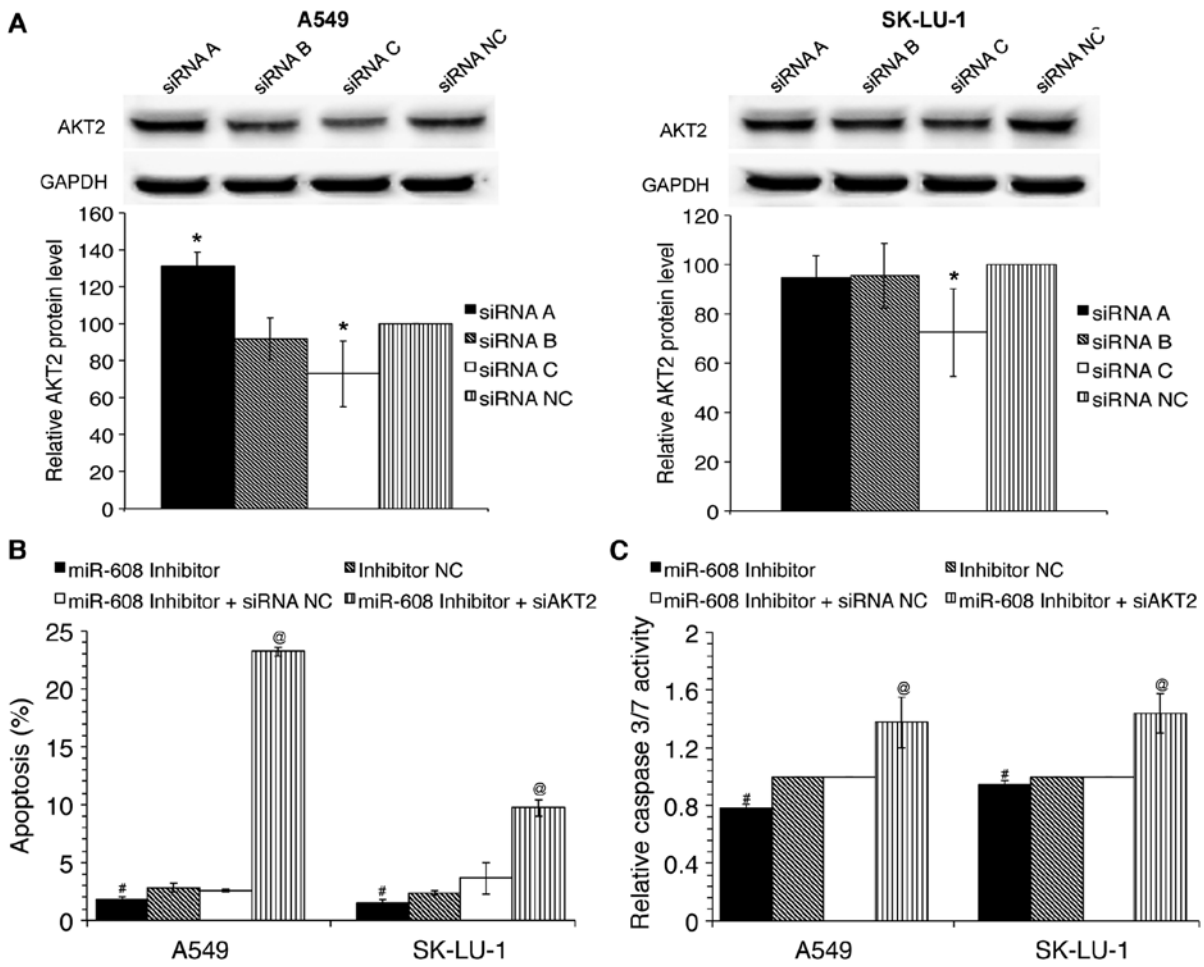

Figure 3. Silencing of AKT2 revives apoptosis inhibited by miR-608 inhibition. (A) Relative AKT2 protein expression following transfection with siRNA duplexes in A549 and SK-LU-1 cells. (B) Detection of apoptosis and (C) caspase-3/7 activity in A549 and SK-LU-1 cells co-transfected with miR-608 inhibitors and si*AKT2*. Statistically significant differences between inhibitor transfected groups and inhibitor NC groups denoted with $^{\#}P\leq0.05$, while statistically significant differences between siAKT2 transfected groups and siRNA NC groups were indicated by $^{@}P\leq0.05$. $^{*}P\leq0.05$.

therefore to evaluate the silencing efficiency of the siRNAs, densitometry analysis of western blot bands was performed to evaluate the AKT2 protein expression in siRNA-transfected in comparison to siRNA NC transfected cells. Amongst the three siRNAs utilized, siRNA C was able to significantly decrease AKT2 protein levels in A549 and SK-LU-1 cells (Fig. 3A). As siRNA C (referred to as si*AKT2* henceforth) had the greatest silencing efficiency amongst the three siRNAs, it was selected for further downstream work. Results indicated that silencing of *AKT2* was able to partially rescue the inhibition of apoptosis and caspase-3/7 activation that was induced by miR-608 inhibitors (Fig. 3B and C). Collectively, these results demonstrate the tumour suppressor role of miR-608 in NSCLC is at least partially through its inhibition of *AKT2*.

Transfection of miR-608 increases caspase-3 detection in zebrafish embryo animal model. The *in vivo* effect of miR-608 on apoptosis was determined through utilization of zebrafish embryos as an animal model. miR-608 mimic, inhibitor or negative control transfected A549 cells were microinjected into zebrafish. Embryos were then visualized using a Leica confocal microscope following immunostaining with anti-active caspase-3 monoclonal antibodies (Fig. 4A). Results of fluorescent image analysis using the ImageJ software indicated that detection of caspase-3 was significantly increased in zebrafish injected with miR-608 in comparison to negative control injected zebrafish (Fig. 4B). This suggests that miR-608 is able to induce apoptosis *in vivo* through caspase activation.

Discussion

While progress has been made in molecular targeted therapies and early diagnosis of lung cancer, the 5-year survival rate of patients is still very low due to most patients being diagnosed at an advanced stage (2). It is therefore essential for identification of novel diagnostic biomarkers and to explore more effective and safe treatment tools. Recent studies have shown that dysregulation of miRNA expression contributes to the development and progression of cancer (26-28). miRNA profiles can also be used as biomarkers for detection of cancer (29-34) and to predict chemotherapeutic response (35-38). It is therefore of particular interest to investigate the therapeutic application of miRNAs in lung cancer.

miR-608 is a novel prognostic marker in carcinogenesis with its expression downregulated in various cancers including chordoma (18), colon cancer (19), glioblastoma (20) and osteosarcoma (21). Single-nucleotide polymorphisms in miR-608 have also been associated with several cancers

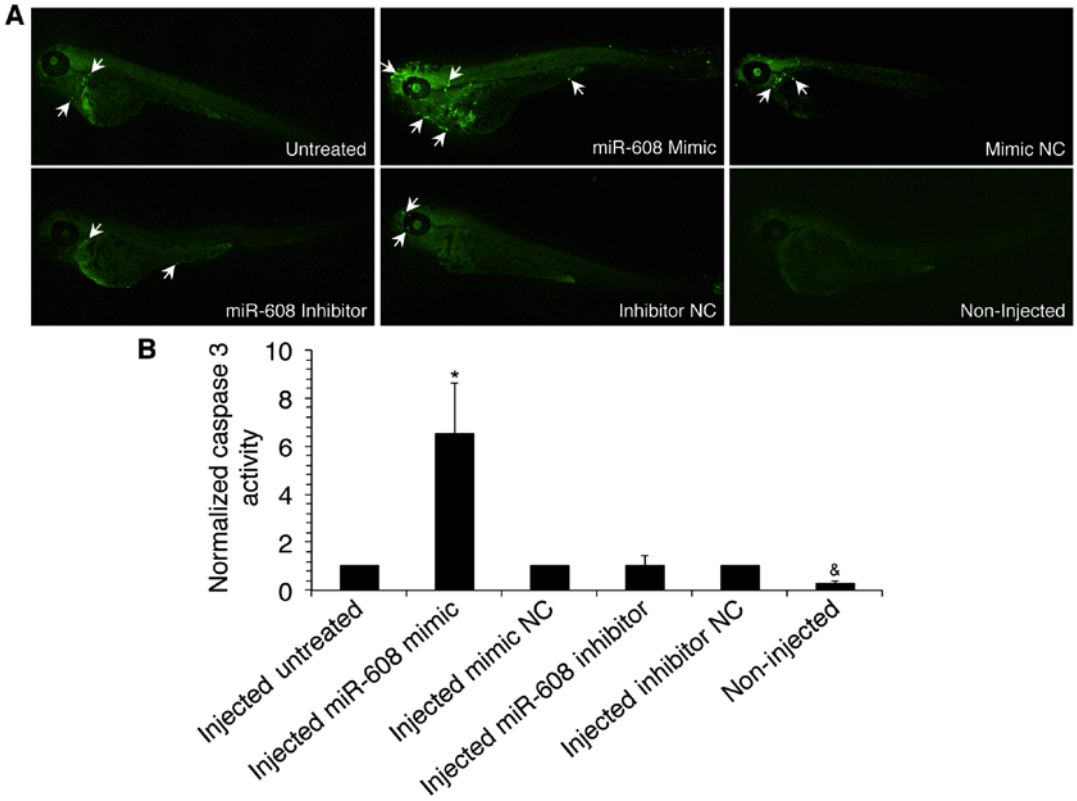

Figure 4. Overexpression of miR-608 induces caspase-3 activation *in vivo*. (A) Examination of zebrafish embryos by confocal microscopy following miR-608 injection. Arrows indicate positive active caspase-3 staining. (B) Fluorescence was quantified and analyzed using ImageJ Analyst software to generate normalized arbitrary fluorescence units. Statistically significant differences between mimic transfected groups and mimic NC groups were indicated by $^{*}P \leq 0.05$. Statistically significant differences between non-injected group and injected untreated groups are denoted with $^{\&}P \leq 0.05$.

such as nasopharyngeal carcinoma (39), colorectal adenocarcinoma (40-42), breast (43,44) and bladder cancer (45). In recent years evidence has emerged illustrating the role miR-608 plays as a tumour suppressor. In chordoma cancer, miR-608 induces apoptosis and inhibits cell proliferation via regulation of epidermal growth factor receptor (*EGFR*) and B-cell lymphoma-extra large (*BCL-XL*) (18). miR-608 has also been shown to directly target macrophage migration inhibitory factor (*MIF*), inhibiting proliferation, migration and invasion, and inducing apoptosis in both osteosarcoma cell lines (21) and glioma stem cells (20). Furthermore, miR-608 has been demonstrated to repress tumorigenesis of colon cancer cells both *in vitro* and *in vivo* through the regulation of N-a-acetyltransferase 10 protein (*NAA10*) (19).

Our previous study revealed that downregulation of anti-apoptotic *BCL-XL* in lung adenocarcinoma cell line A549 resulted in a decrease in cell proliferation, an increase in apoptosis as well as dysregulation of various miRNAs, including upregulation of miR-608. It was further demonstrated via overexpression and knockdown studies that miR-608 plays a role in *BCL-XL* induced apoptosis in A549 cells (22). To identify the molecular mechanism by which miR-608 regulates apoptosis in NSCLC cells, in the present study bioinformatics analysis was performed, which predicted *AKT2* as a novel target with two regions containing perfect complementary miR-608 binding sites in its 3'UTR. Measurement of relative firefly luciferase activity, indicative of translation from the plasmid, and quantification of protein levels via western blot analysis validated *AKT2* as a direct target of miR-608.

AKT2 is a serine/threonine protein kinase that plays an essential role in various signaling pathways including metabolism, proliferation, cell survival, growth and angiogenesis (45,46). Increasing evidence suggests that hyperactivation of AKT2 plays an important role in human malignancy, with amplification and overexpression being reported in several cancers including breast (47,48), pancreatic (49), hepatocellular (50), ovarian (51,52), thyroid (53), glioma (54,55), colorectal (55) and non-small cell lung cancer (56-58). AKT2 has been reported to play a role in cell cycle progression in breast cancer cell line MDA-MB-231, with silencing of *AKT2* leading to cell cycle arrest through downregulation of Cdk2 and cyclin D and upregulation of p27. Prolonged inhibition of *AKT2* was also shown to lead to an increase in the mitochondrial volume, eventually leading to cell death by autophagy (48). Another study indicates that silencing of *AKT2* in neuroblastoma disrupts cell migration and invasion and also decreases metastasis in the liver (59). Downregulation of *AKT2* has also been demonstrated to lead to MCL-1 cleavage, collapse of the mitochondrial membrane potential, release of cytochrome *c* into the cytosol, followed by activation of the caspase cascade in NSCLC (56). Similarly, in glioma cell lines, knockdown of *AKT2* was able to induce apoptosis via dephosphorylation of BAD and the activation of caspase-9 and caspase-3 (55).

As *AKT2* is a well-established pro-survival factor, we hypothesized that targeting of *AKT2* could be a mechanism by which miR-608 functions as a tumour suppressor in NSCLC. This was further validated when cells were co-transfected with miR-608 inhibitors and si*AKT2* to partially rescue inhibition

of apoptosis induced by miR-608 inhibitors. In a recently published study, the relationship between miR-608 and the AKT pathway in bladder cancer further supports the tumour suppressive role of miR-608. Liang and colleagues (60) demonstrated that upregulation of miR-608 was able to suppress cell cycle progression through direct inhibition of *FLOT1* 3'UTR, which is an upstream regulator of the AKT/FOXO3a signaling pathway.

To summarize, in the present study we identified a tumour suppressive role of miR-608 in non-small cell lung cancer (NSCLC). Its role as a tumour suppressor was attributed to identification of a novel direct target, *AKT2*. *In vivo* studies using the zebrafish animal model also confirmed that miR-608 could significantly induce activation of caspase-3, a major apoptotic effector. *AKT2* has been illustrated to have significant roles in tumour progression; therefore selective targeting of *AKT2* via miR-608 may be developed and used as a strategic treatment for NSCLC cancer.

Acknowledgements

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