MicroRNA-345 induces apoptosis in pancreatic cancer cells through potentiation of caspase-dependent and -independent pathways

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Background: Previously, miR-345 was identified as one of the most significantly downregulated microRNAs in pancreatic cancer (PC); however, its functional significance remained unexplored.

Methods: miR-345 was overexpressed in PC cells by stable transfection, and its effect on growth, apoptosis and mitochondrial-membrane potential was examined by WST-1, Hoechst-33342/Annexin-V, and JC-1 staining, respectively. Gene expression was examined by quantitative reverse-transcription-PCR and/or immunoblotting, and subcellular fractions prepared and caspase-3/7 activity determined by commercially available kits. miR-345 target validation was performed by mutational analysis and luciferase-reporter assay.

Results: miR-345 is significantly downregulated in PC tissues and cell lines relative to normal pancreatic cells, and its expression decreases gradually in PC progression model cell lines. Forced expression of miR-345 results in reduced growth of PC cells because of the induction of apoptosis, accompanied by a loss in mitochondrial membrane potential, cytochrome-c release, caspases-3/7 activation, and PARP-1 cleavage, as well as mitochondrial-to-nuclear translocation of apoptosis-inducing factor. These effects could be reversed by the treatment of miR-345-overexpressing PC cells with anti-miR-345 oligonucleotides. BCL2 was characterised as a novel target of miR-345 and its forced-expression abrogated the effects of miR-345 in PC cells.

Conclusions: miR-345 downregulation confers apoptosis resistance to PC cells, and its restoration could be exploited for therapeutic benefit.

Pancreatic cancer (PC) is one of the deadliest malignancies. With an estimated 48 960 diagnoses and nearly 40 560 deaths in 2015, it is considered the fourth leading cause of cancer-related death in the United States (Siegel et al, 2015). Owing to the lack of specific symptoms, early detection is not possible and most PCs are diagnosed in advanced and metastatic stage, leaving limited options for therapy (Feldmann and Maitra, 2008). Most current therapies provide no or marginal survival benefits and therefore 5-year overall survival rate of PC patients has stayed at 3–6% for past few decades (Arora et al, 2013). Clearly, there is an urgent need to identify novel diagnostic/therapeutic targets, understand the molecular mechanisms underlying the aggressive nature of this
malignancy, and develop alternative strategies and novel therapeutics for effective treatment of this devastating disease.

MicroRNAs (miRNAs) are small (~22-nucleotide) noncoding RNAs that act as important regulators of gene expression (Bhardwaj et al., 2010; Srivastava et al., 2013). miRNAs act by specifically binding to the 3′ untranslated region (UTR) of their cognate target mRNAs and subsequently cause either their degradation or inhibit translation (Bhardwaj et al., 2010). To date, ~2469 miRNAs have been identified in humans (Friedlander et al., 2014; Zearo et al., 2014), and emerging evidence suggests that miRNAs potentially regulate ~30–80% of all protein-coding genes (Lu and Clark, 2012). These miRNAs control various key cellular processes, such as proliferation, apoptosis, differentiation, and development, and are implicated in several human diseases, including cancer (Bhardwaj et al., 2010; Ryan et al., 2010). Earlier, it was demonstrated that ~52.5% of miRNA genes are located in cancer-associated genomic regions, or in fragile sites, thus strongly suggested their role in cancer (Calin et al., 2004). Depending on the miRNA targets, miRNAs may function either as oncogenes (by inhibiting tumour suppressor genes) or as tumour suppressors (by inhibiting oncogenes) and thus have an important role in the progression and pathogenesis of cancer (Bhardwaj et al., 2010). According to recently published studies, a number of miRNAs have been shown to be differentially expressed in PC (Srivastava et al., 2014). These aberrantly expressed miRNAs have diverse pathological functions, such as regulating cell cycle progression, apoptosis, chemoresistance, angiogenesis, tumorigenicity, and metastatic potential (Srivastava et al., 2014). miR-345 was reported to be one of the top aberrantly expressed miRNAs in PC exhibiting significant downregulation (Bloomston et al., 2007; Lee et al., 2007; Szafranska et al., 2007); however, its role in PC pathogenesis has remained unexplored thus far.

In the current study, we demonstrate that miR-345 is significantly downregulated in PC, and its restoration in PC cells diminishes growth and induces apoptosis through activation of caspase-dependent and -independent pathways. Moreover, we show that miR-345 directly targets the 3′UTR of BCL2 and suppresses its expression at the protein level. Furthermore, we present evidence that the unopposed expression of BCL2 leads to abrogation of pro-apoptotic effects of miR-345. Together, these findings highlight the pro-apoptotic function of miR-345 in PC and support its utility as a candidate for diagnostic and therapeutic target.

**MATERIALS AND METHODS**

**Cell culture and pancreatic tissue specimens.** Human PC cell lines (MiaPaCa, Panc1, Colo-357, HPAF, ASPC-1, Panc1.05, Panc02.03, Panc03.27, BXPC3, CPIF1, CAPAN1, and SW1990) were maintained as monolayer cultures in DMEM or RPMI 1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) and 100 μg of penicillin and 100 μg ml−1 of streptomycin (Invitrogen). The hTERT-HPNE cells were maintained in Medium D containing 1 volume of M3 base (Cell Corp., San Antonio, TX, USA), 3 volumes of glucose-free DMEM, 5.5 mM glucose, 10 ng ml−1 epidural growth factor, 50 μg ml−1 gentamicin (all from Sigma-Aldrich, St Louis, MO, USA), and 5% fetal bovine serum. All the PC cell lines were cultured in humidified atmosphere at 37 °C with 5% CO2 and media were replaced as needed. Cell lines were tested intermittently for mycoplasma contamination. Frozen pancreatic tissue (normal and malignant) specimens were obtained through the cooperative human tissue network at the University of Alabama at Birmingham under an institutional review board-approved protocol.

**Plasmids and transfections.** For the generation of stably miR-345-overexpressing cell lines, MiaPaCa and Panc1 cells were transfected with miR-345 expression (pCMV-miR-345) or control (pCMV-Neo) plasmid (Origene, Rockville, MD, USA) using X-tremeGENE HP DNA transfection reagent (Roche, Indianapolis, IN, USA) as per the manufacturer’s instructions. Stable populations of transfected cells were selected in media containing 200 μg ml−1 of G418 (Gibco, Grand Island, NY, USA), expanded and examined for stable miR-345 overexpression. For inhibition of miR-345 function, miR-345-overexpressing cells (Panc1-miR-345 and MiaPaCa-miR-345) were transiently transfected with anti-miR-345 or miRNA inhibitor negative controls (Ambion, Austin, TX, USA) at 30 nM concentration using X-tremeGENE HP DNA transfection reagent. For BCL2 overexpression, cells were transiently transfected with pcDNA3-BCL2 construct (lacking 3′UTR region) generated by Stanley Korsmeyer Laboratory (procured through Addgene, Cambridge, MA, USA, plasmid # 8768) or pcDNA3 (Invitrogen) as a control vector using X-tremeGENE HP DNA transfection reagent.

**RNA isolation and real-time quantitative reverse-transcription PCR (qRT-PCR).** Total RNA was extracted using TRIzol reagent as per the manufacturer’s instructions (Invitrogen). Complementary DNA (cDNA) was synthesised using the high-capacity cDNA reverse transcription kit following manufacturer’s instructions (Applied Biosystems, Carlsbad, CA, USA). Expression level of mature miR-345 was examined by following the strategy as described earlier (Srivastava et al., 2011). To examine the expression level of BCL2, real-time qRT-PCR was performed using 96-well plates using cDNA and SYBRGreen Master Mix (Roche) on an iCycler system (Bio-Rad, Hercules, CA, USA) with specific primers. Threshold cycle (Ct) values for miR-345 and BCL2 were normalised against Ct values for U6 small nuclear RNA and GAPDH, respectively, and a relative fold change in expression was calculated. Details of the primers used in the study are provided in Supplementary Table 1. The PCR conditions used were: cycle 1: 95 °C for 10 min, cycle 2 (x40): 95 °C for 10 s and 58 °C for 45 s.

**Cell growth assays.** PC cells (5 × 103 cells per well) were seeded in 96-well plates, and cultured up to 5 days. Media were replaced every day with fresh complete media. Cell growth was monitored each day using WST-1 assay kit (Roche Diagnostics, Mannheim, Germany) as described earlier (Bhardwaj et al., 2014; Deshmukh et al., 2015). Separately, miR-345-overexpressing cells were transiently transfected with BCL2 for 24 h, cultured up to 5 days and growth monitored as described above.

**Hoechst 33342 staining.** Cells (2.5 × 105) were grown in glass-bottom fluorodishes, washed with PBS, and incubated with 10 mg ml−1 Hoechst 33342 (Life Technologies, Grand Island, NY, USA) in the dark at room temperature for 10 min. Cells were then observed and photographed using a Nikon Eclipse TE2000-U fluorescent microscope (Nikon Instruments Inc, Melville, NY, USA).

**Apoptosis assay.** Cells (1 × 106) were seeded in to six-well plates. After 24 h, culture medium was replaced with fresh complete medium and cells were allowed to grow for next 48 h. Subsequently, the extent of apoptosis was measured as previously described (Srivastava et al, 2012; Arora et al, 2015). In brief, cells were harvested and stained with 7-amino-actinomycin (7-AAD) and PE Annexin V using commercially available kit (BD Pharmingen, San Diego, CA, USA) followed by flow cytometry on a BD-FACS Canto II (Becton-Dickinson, San Jose, CA, USA). Percentage of the apoptotic cells was calculated using DIVA software version 6.1.3 (Becton-Dickinson).

**Mitochondrial membrane potential (ΔΨm) determination.** Cells (1 × 106) grown in six-well plates were treated with 20 μg ml−1 of JC-1 dye (Life Technologies) for 20 min and incubated at 37 °C in the dark. Following incubation, cells were harvested by
trypsinising, washed, resuspended in 500 μl of PBS, and analysed by flow-cytometry on a BD-FACS Canto II. Percentage of loss in Δψm was calculated using DIVA software version 6.1.3.  

**Subcellular fractionation.** The cytoplasmic, mitochondrial, and nuclear fractions from low- and high-miR-345-expressing PC cells were prepared using commercially available kit as per the manufacturer’s protocol (Mitosciences, Oregon, OR, USA).  

**Western blot analysis.** Protein isolation and western blotting were performed as described earlier (Srivastava et al, 2011; Tyagi et al, 2014). Immunodetection was carried out using specific antibodies (1:1000) against BCL2, cytochrome c, cleaved caspase-3, cleaved caspase-7, PARP-1, apoptosis-inducing factor (AIF; rabbit monoclonal), HDAC (mouse monoclonal; Cell Signalling, Danvers, MA, USA), z-tubulin (rabbit monoclonal), and VDAC (goat monoclonal; Santa Cruz Biotechnology, Dallas, TX, USA). β-Actin (mouse monoclonal: 1: 20000; Sigma-Aldrich, St Louis, MO, USA), z-tubulin, VDAC, and HDAC served as an control for total, cytoplasmic, mitochondrial, and nuclear fractions, respectively. All secondary antibodies (Santa Cruz Biotechnology) were used at 1: 2500 dilution. Proteins were visualised with the SuperSignal West Femto Maximum sensitivity substrate kit (Thermo Scientific, Logan, UT, USA) and the signal detected using an LAS-3000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

**Dual luciferase 3’UTR-reporter assay.** For the validation of BCL2 as a direct target of miR-345, cells were transiently co-transfected for 24 h with 200 ng of pLuc3U-BCL2 target-reporter plasmid containing BCL2 3’UTR region (Signosis, Santa Clara, CA, USA) along with 0.25 μg of control reporter plasmid (pRL-TK; Promega); plasmid containing a Renilla reniformis luciferase gene downstream of the thymidine kinase (TK) promoter. Moreover, as a control, we also generated a mutant BCL2 3’UTR (MUT-BCL2 3’UTR) reporter construct by site-directed mutagenesis in the putative target region of miR-345 using Quickchange XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) and transiently transfected as described above. After 48 h of transfection, cells were harvested in reporter lysis buffer (Promega). Firefly and Renilla luciferase activities were measured using a dual-luciferase assay kit (Promega) according to the manufacturer’s instructions. The data are represented as the ratio of firefly to Renilla luciferase activity.

**Statistical analysis.** All the experiments were performed at least three times and numerical data expressed as mean ± s.d. The expression profiles of miR-345 in malignant pancreatic versus normal tissues were analysed using unpaired one-tailed Student’s t-test. A pairwise comparison was performed to check if miR-345 had lower expression level in PCs than in NPs. A value of \( P < 0.05 \) was considered to be significant throughout the study.

**RESULTS**

**miR-345 is downregulated in PC.** We examined the expression of miR-345 in a set of normal \((n = 7)\) and cancerous \((n = 21)\) pancreatic tissues, in an in vitro progression model cell lines (hTERT-HPNE and derived lines; Campbell et al, 2008) and a panel of established PC cell lines. Our data reveal that miR-345 is downregulated in the majority of PC cases as compared with the normal pancreas. Relative mean expression of miR-345 in the normal pancreatic tissues is significantly higher (41.93, \( P \)-value<0.001) than that of the malignant pancreatic tissues (11.85; Figure 1A). Furthermore, we observe that the expression of miR-345 is lost gradually in PC progression model cell lines (Figure 1B); whereas a differential expression pattern is seen among various established PC cell lines (Figure 1C). Notably, expression of miR-345 is relatively downregulated in poorly differentiated cell lines as compared with well-differentiated cell lines (Figure 1C). Altogether, our data demonstrate the downregulated expression of miR-345 in PC.

**miR-345 promotes apoptosis in PC cells.** To investigate the biological relevance of miR-345 downregulation in PC, we stably overexpressed it in two poorly differentiated PC cell lines, Panc1 and MiaPaCa (exhibiting low endogenous expression of miR-345), miR-345 overexpression was confirmed by real-time RT-PCR analysis. The data show that Panc1-miR-345 and MiaPaCa-miR-345 cells have ~63- and ~51-fold overexpression of miR-345, respectively, as compared with the control cell lines (Supplementary Figure 1). Growth analyses of miR-345-transfected cells revealed ~57.8% and ~52.6% reduction in overall growth by 5th day in Panc1-miR-345 and MiaPaCa-miR-345, respectively, relative to control vector (Panc1-Neo and MiaPaCa-Neo)-transfected cell lines (Figure 2A). Morphological examination demonstrated that miR-345-overexpressing PC cells became round and shrunken, and many of them detached from the bottom of the culture plate (data not shown). Therefore, we next examined the effect of miR-345 overexpression on chromatin condensation and nuclear fragmentation by Hoechst 33342 staining to confirm if the reduced growth and morphological alterations resulted from cellular apoptosis. Data demonstrate the presence of cells with fragmented nuclei and condensed chromatin in miR-345-overexpressing PC cells (Figure 2B), suggesting apoptosis induction upon restoration of miR-345 in PC cells. We then confirmed apoptosis induction by performing Annexin V and 7-AAD staining followed by flow cytometry. The data reveal a higher apoptotic index (Annexin V-positive/7-AAD-negative cells) in Panc1-miR-345 (48.5%) and MiaPaCa-miR-345 (36.5%) cells, as compared with their respective control cells, that is, Panc1-Neo (21.2%) and MiaPaCa-Neo (17%); Figure 2C). Importantly, the effect of miR-345 restoration on apoptosis was abolished following treatment of miR-345-overexpressing PC cells with anti-miR-345 (Figure 2C). Taken together, these results indicate that miR-345 promotes apoptosis in PC cells.

**miR-345 activates mitochondrial pathways of apoptosis in PC.** Mitochondria are known to have a central role in apoptosis (Elkholi et al, 2014; Bhat et al, 2015). Therefore, we examined the effect of miR-345 on the mitochondrial membrane potential (Δψm), and localisation/activation of the key proteins involved in apoptosis. Our flow cytometry analysis revealed a high proportion (35.6% and 29.2%, respectively) of damaged mitochondria/loss of Δψm in miR-345-overexpressing Panc1 and MiaPaCa cells as compared with their respective controls (Figure 3A). Moreover, we observed that the loss of Δψm was associated with increased levels of cytochrome c in the cytosol with a concomitant decrease in the mitochondria of miR-345-overexpressing cells (Figure 3B). Similarly, we also observed increased levels and activity of effector caspases (cleaved caspases-3 and -7) (Figure 3C and Supplementary Figure 2) along with PARP-1 cleavage in miR-345-overexpressing PC cells (Figure 3C). Interestingly, the effects of miR-345 overexpression on Δψm, cytochrome c translocation, and activation of caspases were attenuated by treatment with miR-345 inhibitor (Figure 3A–C). To explore the possibility of caspase-independent apoptosis, we examined the levels of AIF, known to induce apoptosis in a caspase-independent manner (Cande et al,
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The recent discovery of miRNAs has revealed a novel mechanism of gene regulation and provided new avenues for cancer research (Srivastava et al., 2014). These miRNAs are highly deregulated in various malignancies, including PC, and their aberrant expression

**Figure 1.** miR-345 is downregulated in pancreatic cancer. (A) miR-345 expression profiling was performed in normal (n = 7) and cancerous (n = 21) pancreatic tissue specimens. A decreased expression of miR-345 is observed in majority of cancer tissues as compared with the normal pancreas. Dark lines indicate mean expression level of miR-345 of the group and the fold reflects the ratio between the mean values of miR-345 expression in normal vs cancer cases. (B) Expression analysis of miR-345 in hTERT-HPNE PC in vitro progression model demonstrates a gradual decrease in the expression of miR-345. (C) A differential expression pattern of miR-345 is observed in PC cell lines. All the poorly differentiated cell lines exhibit relatively lower expression as compared with the well-differentiated cell lines. U6 small nuclear RNA served as an internal control. A relative quantity of miR-345 was determined using the $2^{-\Delta\Delta CT}$ method. Bars represent the mean ± s.d. (n = 3).

**DISCUSSION**

The data reveal that the level of nuclear AIF is increased with a concomitant decrease in its level in the mitochondrial fraction in miR-345-overexpressing Panc1 and MiaPaCa cells (Figure 3D). Furthermore, we observe that the level of mitochondrial AIF is regained in miR-345-overexpressing cells when they are treated with anti-miR-345 (Figure 3D). Together, our findings suggest that miR-345 induces apoptosis in PC cells by promoting caspase-dependent as well as -independent pathways of apoptosis.

miR-345 negatively regulates BCL2 through direct binding to its 3'UTR. To identify the target of miR-345, we performed *in silico* analysis using the algorithms Target Scan (http://www.targetscan.org) and miRanda (http://www.microrna.org), and identified BCL2, an important molecule of the anti-apoptotic gene family, as putative target of miR-345. Further investigation revealed the presence of a 8-mer binding site for miR-345 in the 3'UTR of the BCL2 transcript (Figure 4A). To validate the potential targeting of BCL2 by miR-345, we examined its expression in a miR-345-overexpressing Panc1 and MiaPaCa cells. Our investigation revealed no change in the expression of BCL2 at the transcript level (Figure 4B; upper panel); however, its expression decreased at the protein level in both Panc1-miR-345 and MiaPaCa-miR-345 cells as compared with their respective control cells (Figure 4B; upper panel), thus suggesting its translational repression by miR-345. To test whether BCL2 is a direct target of miR-345, control and miR-345-overexpressing PC cells were transiently transfected with a luciferase reporter plasmid containing a region of BCL2 3'UTR having a wild-type or mutated miR-345 target site (Figure 4C). As shown in Figure 4D, our data demonstrate that miR-345 significantly suppressed the luciferase activity of the reporter plasmid with wild-type-BCL2 3'UTR in Panc1-miR-345 and MiaPaCa-miR-345 (~69% and ~83%, respectively) as compared with that in control cells.

Furthermore, cells transfected with mutated-3'UTR did not show any response to the suppressor activity of miR-345 (Figure 4D). Altogether, our data suggest that BCL2 is a direct target of miR-345.

BCL2 is involved in the miR-345-mediated activation of apoptotic pathways in PC cells. Following identification of BCL2 as a direct target of miR-345, we further examined its significance in miR-345-mediated induction of apoptosis of PC cells. For this, expression vector of BCL2, which encodes the entire coding sequence of BCL2, but lacks the 3'UTR, was transiently transfected into the miR-345-overexpressing PC cells (Panc1-miR-345 and MiaPaCa-miR-345), and the effects on proteins associated with apoptosis pathways were analysed. Our immunoblot analysis shows that forced expression of BCL2 efficiently blocked the miR-345-induced activation of caspases, cleavage of PARP-1, and prevents the nuclear translocation of AIF (Figure 5A). Furthermore, we also examined the effect of BCL2 overexpression on the miR-345 decreased growth of PC cells. Our data demonstrate that forced expression of BCL2 abrogated the growth inhibitory effect of miR-345 in both Panc1 and MiaPaCa cells (Figure 5B). Together, these data suggest that miR-345 promotes apoptosis by suppressing the expression of BCL2, which triggers the mitochondrial pathway of apoptosis (Figure 6).
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Figure 2. miR-345 restoration induces apoptosis in PC cells. (A) Cell growth was monitored by WST-1 assay daily up to 5 days. Data are presented as fold change in comparison to the cell growth on day 1. Data (mean ± s.d., n = 3; *P < 0.05) show decreased growth of miR-345-overexpressing PC cells relative to their respective controls. (B) PC cells were cultured up to 48 h in glass bottom fluoroes, stained with Hoechst 33342, and examined and photographed using a fluorescent microscope. miR-345-overexpressing PC cells show apoptotic characteristics as evident by condensed (arrowhead) and fragmented (arrow) nuclei. Micrographs represent random fields of view (magnification × 400). (C) miR-345-overexpressing PC cells along with their control cells were seeded in six-well plate and media replaced after 24 h. After next 48 h incubation, cells were washed, stained with PE Annexin V, and analysed for apoptosis by flow cytometry. In parallel, miR-345-overexpressing PC cells were transiently transfected with anti-miR-345 or non-target (NT) sequence control, cultured up to 48 h and processed for apoptosis analysis. Histograms are representative of three independent experiments (Panc1, upper panel; MiaPaCa, lower panel). Data suggest a pro-apoptotic role of miR-345 in PC cells.
miR-345 promotes apoptosis through mitochondrial pathways. (A) miR-345-overexpressing PC (Panc1-miR-345 and MiaPaCa-miR-345) cells were transiently transfected with anti-miR-345 or non-target (NT) sequence control. After 48 h of transfection, cells were stained with JC-1 as described in Materials and Methods and subjected to flow cytometry. Dot plot shows cell population with intact mitochondria (having high ΔΨm; in blue); and population with damaged mitochondria (having low ΔΨm; in orange). Results demonstrate a loss of ΔΨm in miR-345-overexpressing PC cells, an effect that was abrogated upon treatment with anti-miR-345. (B-D) Total, cytoplasmic, nuclear, or mitochondrial protein fractions were collected from low- and high-miR-345-expressing PC cells; and high-miR-345-expressing cells transiently transfected with anti-miR-345 or NT sequence control. Thereafter, expression of (B) cytochrome c (in cytosolic and mitochondrial fractions), (C) cleaved caspase-3, caspase-7, and PARP-1 (in total fraction) and (D) AIF (in nuclear and mitochondrial fractions) was examined by immunoblot assay. β-Actin, α-tubulin, VDAC, and HDAC were used as loading control for the total, cytosolic, mitochondrial, and nuclear fractions, respectively.

(ΔΨm) is the important event for the mitochondria-mediated apoptosis pathways (Gupta et al, 2009). Following the collapse of ΔΨm, cytochrome c is released into the cytosol, where it forms complex with apoptotic protease-activating factor-1 and ATP. This complex then binds to pro-caspase-9, and causes its cleavage, which further initiates the activation of effector caspase-3 and -7. These effector caspases then cleave PARP-1, a known cellular substrates of caspases, whose cleavage by caspases is considered to be a characteristic of caspase-dependent apoptosis (Elmore, 2007). Along these lines, we demonstrated a disruption of ΔΨm in PC cells following miR-345 overexpression, release of cytochrome c from mitochondria to cytosol, and further activation of effectors caspase-3, caspase-7, and subsequent PARP-1 cleavage. Although caspase activation is considered a hallmark of apoptotic cell death, mitochondrially mediated apoptosis induction also occurs in a caspase-independent manner (Borner and Monney, 1999; Sperandio et al, 2000). AIF is localised to the mitochondrial intermembrane space, and upon apoptotic stimuli, AIF translocates to the nucleus where it causes chromatin condensation and nuclear fragmentation, resulting in cell death (Cande et al, 2004). Interestingly, in our study, we found that miR-345 overexpression induced the translocation of AIF to the nucleus. Thus, miR-345-induced apoptosis involved both cytochrome c (caspase-dependent) and AIF (caspase-independent) in the mitochondrial apoptotic pathway.

Findings from our study are quite interesting as we identified anti-apoptotic BCL2 to be a direct target of miR-345. Our study suggested that miR-345 downregulation-mediated upregulation of BCL2 could be a key event in triggering apoptosis resistance in PC cells. Several lines of evidence show that BCL-2 is overexpressed in variety of malignancies, including PC (Bold et al, 2001; Sun et al, 2002; Scherr et al, 2014), and its elevated levels have been shown to be associated with apoptosis-resistance and enhanced metastatic potential in PC (Bold et al, 1999, 2001). BCL2 keeps a check on the release of cytochrome c and AIF from mitochondria by preventing the disruption of mitochondrial membrane potential (Susin et al, 1996; Harris and Thompson, 2000; Tait and Green, 2010). We demonstrated that exogenous expression of BCL2 abrogated the miR-345-induced activation of caspases and prevented the translocation of AIF to the nucleus, favouring cell survival. Yang et al (1997) in their study investigated that overexpression of BCL2 prevents the initiation of the cellular apoptotic programme by
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Figure 4. miR-345 suppresses BCL2 expression in PC cells through directly targeting its 3’UTR. (A) In silico analysis (using algorithms of Target Scan and miRanda) showing miR-345-binding sites in BCL2 3’UTR. (B) Total RNA and protein from control and miR-345-overexpressing PC cells were isolated and expression of BCL2 was examined by qRT-PCR and immunoblot analysis. GAPDH and β-actin served as internal controls for RT-PCR and immunoblot analysis, respectively. Data show that expression of BCL2 remained unaltered at the transcript level, but was repressed at the protein level in both Panc1-miR-345 and MiaPaCa-miR-345 cells. (C) Schematic representation of BCL2 3’UTR with either wild-type (WT) or mutant (MUT) miR-345 target site. MUT 3’UTR construct carries three nucleotides variation in the seed matching region of the target site to disrupt binding of miR-345. (D) PC cells were grown in six-well plate and transiently co-transfected with the luciferase promoter-reporter plasmids containing WT or MUT BCL2-3’UTR along with pRL-TK construct (transfection efficiency control) for 24 h. Thereafter, total protein was collected in passive lysis buffer and subsequently firefly/Renilla luciferase activities were measured using a dual-luciferase assay system. Data (mean ± s.d.; n = 3) are presented as fold change in normalised luciferase activity. *P < 0.05. Results show that miR-345 suppresses the luciferase activity of the reporter plasmid containing WT 3’UTR of BCL2.

Figure 5. miR-345-mediated downregulation of BCL2 activates apoptosis. (A) miR-345-overexpressing PC cells were transiently transfected with BCL2-overexpressing (pcDNA3-BCL2) or control (pcDNA3) plasmids. After 48 h of transfection, total, nuclear, and mitochondrial fractions were collected and effect on the expression of BCL2, cleaved caspases-3 and -7, and cleaved PARP-1 (in total) and AIF (in nuclear and mitochondrial) was examined by immunoblot analysis using specific antibodies. β-Actin, VDAC, and HDAC were used as loading control for the total, mitochondrial, and nuclear fractions, respectively. Data demonstrate that forced expression of BCL2 blocked the miR-345-induced activation of caspases, PARP-1, and prevents the nuclear translocation of AIF. (B) Panc1-miR-345 and MiaPaCa-miR-345 cells were transiently transfected with BCL2-overexpressing or control plasmids. After 24 h of transfection, cells were trypsinised, counted, and seeded (5 × 10^3 per well) in 96-well plate. Thereafter, cell growth was monitored by WST-1 assay daily up to 5 days. Data (mean ± s.d.; n = 3) presented as fold change as compared with the cell growth on day 1. *P < 0.05. Results show that forced expression of BCL2 abrogated the growth inhibitory effect of miR-345 in PC cells.

blocking the release of cytochrome c from mitochondria. Moreover, a separate study by Susin et al (1998) demonstrated that BCL2 interferes with permeability transition pores and prevents the release of AIF. In this regard, the ability of miR-345 to downregulate the anti-apoptotic gene BCL2 is highly significant.
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Figure 6. The proposed mechanistic model of miR-345-induced apoptosis in PC cells. The anti-apoptotic BCL2 protein guards the outer mitochondrial membrane and controls apoptosis by maintaining the mitochondrial permeability. miR-345 inhibits BCL2 through its direct binding, and its inhibition result in the loss of ΔΨm, which allows cytochrome c to be released from the mitochondria into the cytosol. Released cytochrome c then binds with apoptotic protease-activating factor-1 and procaspase-9, forms apoptosis, activates caspases-9 and -3/7 and PARP1 cleavage, and causing apoptosis. At the same time, Apaf1 and procaspase-9, forms apoptosome, activates caspases-9 Released cytochrome c to be released from the mitochondria into the cytosol. 

In summary, we showed that downregulation of miR-345 was a frequent event in PC, and this downregulation significantly correlated with PC progression. Furthermore, ectopic expression of miR-345 in PC cells dramatically reduced cell growth and induced apoptosis. We further identified BCL2 as functional target of miR-345, and proved the involvement of the mitochondrial pathway in miR-345-promoted apoptosis. Our findings thus suggest an essential role of miR-345 in regulating apoptosis, and implicate the potential application of miR-345 in PC therapy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Arora S, Bhardwaj A, Singh S, Srivastava SK, McClellan S, Nirodi CS, Piazza GA, Grizzle WE, Owen LB, Singh AP (2013) An undesired effect of chemotherapy: gemcitabine promotes pancreatic cancer cell invasiveness through reactive oxygen species-dependent, nuclear factor kappab- and hypoxia-inducible factor Alpaha-mediated up-regulation of CXCR4. J Biol Chem. 288(29): 21197–21207.

Bauer C, Hees C, Sterzik A, Bauerfeind F, Mak’Anyego R, Duwell P, Lehr HA, Noesner E, Wank R, Trauzold A, Endres S, Dauer M, Schnurr M (2015) Proapoptotic and Antianpoptotic Proteins of the Bcl-2 Family Regulate Sensitivity of Pancreatic Cancer Cells Toward Gemcitabine and T-Cell-Mediated Cytotoxicity. J Immunother 38(3): 116–126.

Bhardwaj A, Singh S, Singh AP (2010) MicroRNA-based cancer therapeutics: big hope from small RNAs. Mol Cell Pharmacol 2(5): 213–219.

Bhardwaj A, Srivastava SK, Singh S, Arora S, Tyagi N, Andrews J, McClellan S, Carter JE, Singh AP (2014) CXCL12/CXCR4 signaling counteracts docetaxel-induced microtubule stabilization via p21-activated kinase 4-dependent activation of LIM domain kinase 1. Oncotarget 5(22): 11490–11500.

Bhat TA, Kumar S, Chaudhary AK, Yadav N, Chandra D (2015) Restoration of mitochondria function as a target for cancer therapy. Drug Discov Today 20(5): 635–643.

Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, Liu CG, Bhattacharya S, Taccioli C, Croce CM (2007) MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA 297(17): 1901–1908.

Bold RJ, Chandra J, McConkey DJ (1999) Gemcitabine-induced programmed cell death (apoptosis) of human pancreatic carcinoma is determined by Bcl-2 content. Ann Surg Oncol 6(3): 279–285.

Bold RJ, Virudachalam S, McConkey DJ (2001) BCL2 expression correlates with metastatic potential in pancreatic cancer cell lines. Cancer 92(5): 1122–1129.

Borner C, Monney L (1999) Apoptosis without caspases: an inefficient molecular guillotine? Cell Death Differ 6(6): 497–507.

Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci USA 101(9): 2999–3004.

Campbell PM, Lee KM, Ouellette MM, Kim HJ, Groehler AL, Khazak V, Der CJ (2008) Bas-driven transformation of human nestin-positive pancreatic epithelial cells. Methods Enzymol 439: 451–465.

Cande C, Vahsen N, Garrido C, Kroemer G (2004) Apoptosis-inducing factor (AIF): caspase-independent and all. Cell Death Differ 11(6): 591–595.

Deshmukh SK, Srivastava SK, Bhardwaj A, Singh AP, Tyagi N, Marimuthu S, Dyss DL, Zotto VD, Carter JE, Singh S (2015) Resistin and interleukin-6 exhibit racially-disparate expression in breast cancer patients, display molecular association and promote growth and aggressiveness of tumor cells through STAT3 activation. Oncotarget 6(13): 11231–11241.

Elkholi R, Renault TT, Serasinghe MN, Chipuk JE (2014) Putting the pieces together: How is the mitochondrial pathway of apoptosis regulated in cancer and chemotherapy? Cancer Metab 6: 2–16.

Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35(4): 495–516.

Feldmann G, Maibra A (2008) Molecular genetics of pancreatic ductal adenocarcinomas and recent implications for translational efforts. J Mol Diagn 10(2): 111–122.

Friedlander MR, Lizano E, Houben AJ, Bezdan D, Banez-Coronel M, Kudla G, Feldmann G, Maitra A (2008) Molecular genetics of pancreatic ductal adenocarcinoma. Cancer 111(10): 2037–2042.

Gallmeier E (2015) Overexpression of heat shock protein 27 (HSP27) regulates the outer mitochondrial membrane permeability. Cell Death Differ 22(7): 1093–1106.

Gao J, Zhu R, Wang X, Yong H, Chen J (2014) Overexpression of LIM domain kinase 1 increases gemcitabine sensitivity in pancreatic cancer cells through STAT3 activation. J Cell Mol Med 18(8): 1763–1773.

Gupta S, Kass GE, Szegedi E, Joseph B (2009) The mitochondrial death pathway: a promising therapeutic target in diseases. J Cell Mol Med 13(6): 1004–1033.

Harris MH, Thompson CB (2000) The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. Cell Death Differ 7(12): 1182–1191.

Lee EE, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL, Morgan DL, Postier RG, Brackett DJ, Schmittgen TD (2007) Expression profiling
identifies microRNA signature in pancreatic cancer. *Int J Cancer* **120**(5): 1046–1054.

Loukopoulos P, Shibata T, Katoh H, Kokubu A, Sakamoto M, Yamazaki K, Kosuge T, Kanai Y, Hosoda F, Imoto I, Ohki M, Inazawa J, Hirohashi S (2007) Genome-wide array-based comparative genomics hybridization analysis of pancreatic adenocarcinoma: identification of genetic indicators that predict patient outcome. *Cancer Sci* **98**(3): 392–400.

Lu J, Clark AG (2012) Impact of microRNA regulation on variation in human gene expression. *Genome Res* **22**(7): 1243–1254.

Lu J, Clark AG (2012) Impact of microRNA regulation on variation in human gene expression. *Genome Res* **22**(7): 1243–1254.

Pogribny IP, Filkowski JN, Tryndyak VP, Golubov A, Shpyleva SI, Kovalchuk O (2010) Alterations of microRNAs and their targets are associated with acquired resistance of MCF-7 breast cancer cells to cisplatin. *Int J Cancer* **127**(8): 1785–1794.

Ryan BM, Robles AL, Harris CC (2010) Genetic variation in microRNA networks: the implications for cancer research. *Nat Rev Cancer* **10**(6): 389–402.

Scherr M, Elder A, Battmer K, Barzan D, Bomken S, Ricke-Hoch M, Schroder A, Venturini I, Blair HJ, Vormoor J, Ottmann O, Ganser A, Pich A, Hilliker-Kleiner D, Heidenreich O, Eder M (2014) Differential expression of miR-17–92 identifies BCL2 as a therapeutic target in BCR-ABL-positive B-lineage acute lymphoblastic leukemia. *Leukemia* **28**(3): 554–565.

Siegel RL, Miller KD, Jemal A (2015) Cancer statistics, 2015. *CA Cancer J Clin* **65**(1): 5–29.

Sperandio S, de Belle I, Bredesen DE (2000) An alternative, nonapoptotic form of programmed cell death. *Proc Natl Acad Sci USA* **97**(26): 14376–14381.

Srivastava SK, Arora S, Singh S, Bhardwaj A, Averett C, Singh AP (2014) MicroRNAs as potential clinical biomarkers: emerging approaches for their detection. *Biotech Histochem* **88**(7): 373–387.

Srivastava SK, Bhardwaj A, Leavesley SJ, Grizzle WE, Singh S, Singh AP (2013) MicroRNAs as potential clinical biomarkers: emerging approaches for their detection. *Biotech Histochem* **88**(7): 373–387.

Srivastava SK, Bhardwaj A, Singh S, Arora S, McClellan S, Grizzle WE, Reed E, Singh AP (2012) Myb overexpression overrides androgen depletion-induced cell cycle arrest and apoptosis in prostate cancer cells, and confers aggressive malignant traits: potential role in castration resistance. *Carcinogenesis* **33**(6): 1149–1157.

Srivastava SK, Bhardwaj A, Singh S, Arora S, Wang B, Grizzle WE, Singh AP (2011) MicroRNA-150 directly targets MUC4 and suppresses growth and malignant behavior of pancreatic cancer cells. *Carcinogenesis* **32**(12): 1832–1839.

Sun CY, Wang BL, Hu CQ, Peng RY, Gao YB, Gu QY, Wang DW (2002) Expression of the bcl-2 gene and its significance in human pancreatic carcinoma. *Hepatobiliary Pancreat Dis Int* **1**(2): 306–308.

Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, Daugas E, Geuskens M, Kroemer G (1996) Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med* **184**(4): 1331–1341.

Susin SA, Zamzami N, Kroemer G (1998) Mitochondria as regulators of apoptosis: doubt no more. *Biochim Biophys Acta* **1366**(1-2): 151–165.

Szafranska AE, Davison TS, John J, Cannon T, Sipos B, Maghnouj A, Labourier E, Hahn SA (2007) MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene* **26**(30): 4442–4452.

Tait SW, Green DR (2010) Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol* **11**(9): 621–632.

Tang JT, Wang JL, Du W, Hong J, Zhao SL, Wang YC, Xiong H, Chen HM, Fang JY (2011) MicroRNA 345, a methylation-sensitive microRNA is involved in cell proliferation and invasion in human colorectal cancer. *Carcinogenesis* **32**(8): 1207–1215.

Tyagi N, Bhardwaj A, Singh AP, McClellan S, Carter JE, Singh S (2014) p19 activated kinase 4 promotes proliferation and survival of pancreatic cancer cells through AKT- and ERK-dependent activation of NF-kappaB pathway. *Oncotarget* **5**(18): 8778–8789.

Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* **275**(5303): 1129–1132.

Zearo S, Kim E, Zhu Y, Zhao JT, Sidhu SB, Robinson BG, Soon PS (2014) MicroRNA-484 is more highly expressed in serum of early breast cancer patients compared to healthy volunteers. *BMC Cancer* **14**: 200–214.

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