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MicroRNA-663 antagonizes apoptosis antagonizing transcription factor to induce apoptosis in epithelial cells

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Abstract

MicroRNAs are small functional RNAs that modulate various biological processes in cells by interfering with gene translation. We have previously demonstrated that certain miRNAs play a crucial role in the innate immune responses of human oral epithelial cells to *Porphyromonas gingivalis*. While addressing the mechanisms of *P. gingivalis* induced apoptosis in these cells, we discovered that certain miRNAs are upregulated upon stimulation with live bacteria. These upregulated miRNAs include hsa-miR-584, hsa-miR-572, hsa-miR-210, hsa-miR-492, hsa-miR-623 and hsa-miR-663. Further analysis revealed an unexpected role for hsa-miR-663 (miR-663). To further evaluate miR-663 function, we overexpressed miR-663 in epithelial cells which resulted in cellular apoptosis. The bioinformatics analysis of the miR-663 target prediction, revealed a strong binding affinity to a 3′ UTR region of Apoptosis Antagonizing Transcription Factor (AATF) mRNA. To demonstrate the binding of miR-663 to AATF mRNA, the putative miR-663 target site within the 3′-UTR region of AATF was cloned in luciferase vector and transfected to HEK293T cells. Luminescence data showed the downregulation of luciferase activity in cells that had the full length target region of the putative binding site, confirming that AATF is one of the targets for miR-663. This prompted us to further evaluate its role in a cancer cell line (MCF-7) to determine miR-663s' apoptotic function. The overexpression of miR-663 led to a significant increase in apoptosis of MCF-7 cells. Taken together, miR-663 may function as an 'apoptomiR' by inhibiting the anti-apoptotic gene AATF to induce apoptosis. These findings could have therapeutic implications for epithelial cell targeting in cancer therapy.

Keywords *Porphyromonas gingivalis* · MicroRNAs · Primary oral epithelial cells · Cell death · ApoptomiRs

Introduction

Porphyromonas gingivalis (*P. gingivalis*), a Gram-negative anaerobic bacteria found in the oral cavity is considered a putative pathogen in periodontitis [1]. *P. gingivalis* is a component of subgingival plaque bacteria that possess numerous virulence factors including factors that aid intracellular invasion, intracellular persistence and host cell apoptosis [2]. We have previously shown that *P. gingivalis* induces apoptosis of primary gingival epithelial cells [2–5]. Apoptosis is a defense mechanism against stressed and infected cells to maintain tissue homeostasis [6]. Apoptosis is prevalent in the gingiva at sites of chronic bacteria-induced inflammation within the connective tissue [7, 8]. Many studies have demonstrated that *P. gingivalis* can modulate apoptosis of various cell types: epithelial cells [9, 10], fibroblasts [11, 12], endothelial cells [13–16] and lymphocytes [17]. Moreover, apoptosis has been proposed as one of the mechanisms to explain the tissue destruction in chronic periodontitis affected tissue. The apoptotic process

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involves the release of a number of intrinsic factors activated by a diverse set of stressors or through receptor mediated extrinsic factors [18]. However, very little is known about the mechanisms of *P. gingivalis* induced apoptosis.

Recent investigations have suggested the involvement of miRNAs in the cellular apoptotic process [19–21]. MiRNAs are a class of short non-coding single-stranded 18–25 nt length functional small RNAs involved in various biological processes [22]. They typically bind to 3′ untranslated regions of messenger RNA (mRNA) and prevent gene expression by translational inhibition or by degradation of mRNA [22]. There are a number of studies demonstrating the roles for miRNAs in modulating the apoptotic process by directly targeting genes involved in both extrinsic as well as intrinsic apoptosis pathways [23]. For example, miR-491 directly targets Bcl-xL to induce apoptosis of human colorectal cancer cells [24], miR-23a/b and miR-27a/b targets Apaf-1 expression leading to apoptosis of neurons [25], miR-204-5p has been shown to inhibit BCL2 to induce apoptosis of prostate cancer cells [26] and overexpression of miR-497 leads to the apoptosis of human ovarian cancer cells by targeting PAX2 mRNA [27]. Numerous reports supported the involvement of miRNAs in cellular apoptosis targeting various proteins such as p53, Caspase-3, BCL-2, CDK6, p27Kip1, BIM etc. [20, 28–32]. Hence, we hypothesized that miRNAs may play a role in the induction of apoptosis mediated by *P. gingivalis* in oral epithelial cells and such knowledge could be biologically and therapeutically relevant to cancer therapy.

In the present study, we evaluated differential miRNA expression and analysis in epithelial cells stimulated with various Toll-like receptor (TLR) ligands, live and heat inactivated *P. gingivalis*, to understand the molecular mechanism of inflammation and apoptosis. We determined that several miRNAs are differentially regulated particularly with live *P. gingivalis* stimulation and hypothesized that these were related to apoptotic mechanisms. Among the up-regulated miRNAs, we chose to further investigate miR-663s' function based on our bioinformatics and ingenuity pathway analysis (IPA), which showed a strong correlation of miR-663 to the cell death pathway. Following further in depth experimentation we now demonstrate miR-663's role in that it induces apoptosis of oral epithelial cells by directly targeting an anti-apoptotic gene called apoptosis antagonizing transcription factor (AATF). These findings may have therapeutic implications in cancer biology.

Materials and methods

Cell culture and challenge assays

We utilized three primary oral epithelial cells from different donors from our inventory of human primary gingival epithelial cells isolated from gingival biopsies as described in our previously publications [4, 5, 22, 33–37]. Defrosted cells were cultured as described previously and cells at 3rd passage were harvested, seeded at a density of 0.5×10^5 cells/well on to six well culture plate coated with type-I collagen, and maintained in 2 ml of complete keratinocyte serum free medium (Invitrogen, CA). When cells reached ~ 90% confluence, they were washed twice with fresh medium and challenged with heat inactivated and live *P. gingivalis* (strain ATCC 33277) at MOI:100, ultrapure lipopolysaccharide (LPS) at 1 µg/ml (Invivogen), Sphingosine-1-Phosphate (S1P) at 100 nM (Enzo Lifesciences), according to our previous reports [5, 38, 39]. MCF-7 cell line was a gift from Dr. Wolfgang Zacharias, University of Louisville, KY.

Bacterial strains and conditions

Porphyromonas gingivalis ATCC 33277 was purchased from the ATCC (Manassas, VA, USA) and grown at low passage in GAM media (Nissui Pharmaceutical, Tokyo, Japan) under anaerobic conditions (85% N_2, 10% CO_2 and 10% H_2; Coy Laboratory) according to [40].

MiRNA array profiling/analysis

Total RNA was collected by the TRIzol method and purified with a Qiagen purification kit (Qiagen, CA) and the quality was analyzed using a Bioanalyzer 2100 (Agilent, CA). Reference pools were generated using equal amounts of each sample. For each array to be hybridized, 2 µg total RNA from each sample and reference pool were labeled with Hy3™ and Hy5™ fluorescent label, respectively, using the miRCURY™ LNA Array labeling kit (Exiqon, Denmark) as previously reporter [22]. Briefly, the Hy3™ labeled sample and the Hy5™ labeled reference pool RNA were mixed and hybridized to the miRCURY™ LNA array version 8.1. The hybridization was performed according to the miRCURY™ LNA array manual using a Tecan HS4800 hybridization station (Tecan, Austria). The miRCURY™ LNA array microarray slides were scanned by a ScanArray 4000 XL scanner (Packard Biochip Technologies, USA) and the image analysis was carried out using the ImaGene 7.0 software (BioDiscovery, Inc., USA). Expression ratios were determined for microarray data by computing the background-corrected fluorescent signal from the query sample (Q)/reference sample (R). Ratiometric data were transformed to log2 to produce a continuous spectrum of up- and down- regulated values. Data were normalized by plotting the difference, log2(Q/R), against the average, (1/2)log2(Q*R) followed by the application of locally weighted regression (lowness) to smooth intensity-dependent ratios. The clustering was performed on log2 (Hy3/Hy5) ratios which passed the filtering criteria on variation across samples with standard deviation

P = 0.50. (column C-T, Expression_matrix) [41]. PCA-plot is performed on log2(Hy3/Hy5) ratios which passed the filtering criteria on variation across samples; standard deviation > 0.50. (column C–T, Expression_matrix).

Real-time PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA). The isolated total RNA sample was converted to cDNA using miR-663 hairpin loop primers (LifeTechnologies, CA). Real-time PCR was performed by using 1 ng of cDNA with miR-663 specific primer and probe on an ABI7500 system (LifeTechnologies, CA). The data was analyzed by normalizing miRNA level to microRNA RNU48 (snoRNA used as internal control which has least variability across the cell types and challenges) [22]. The fold increase was calculated according to the ΔΔCT method [42].

Western blot analysis

Cells were washed with cold PBS and then lysed on ice for 30 min in 100 µl RIPA lysis buffer (Sigma-Aldrich, St. Louis) containing protease (Roche, IN) and phosphatase inhibitors (Sigma-Aldrich, St. Louis). All Western blotting reagents were procured from Invitrogen, CA except for the ECL Plus western blotting detection system (GE Health Care, NJ). PARP (#9542) and Actin antibodies were procured from from Cell Signaling Technologies, AATF or Che-1 antibody (AATF2B6;sc-81225) was purchased from Santa Cruz Biotechnology. The western blotting was performed according to our method [33]. Probing and visualization of immunoreactive bands were performed as previously described [33].

Cloning and transfection

The pre-hsa-miR-663 was cloned into multiple cloning site of pmiRNA1 (pCDH-CMV-MCS-EF1-copGFP) plasmid (Systembiosciences, CA). MCF-7 cells (human breast cancer cell line) were grown in optiMEM containing 0.01 µg ml insulin and 1 ml penn-strep/100 ml. The cells were detached and seeded into a six well plate at 0.5×10^5 cells/well. The next day, the cells were transfected with either mock (pmiRNA-1-GFP alone) or pmiRNA1-663 using Fugene-6 transfecting agent. 2 µg of plasmid/well was diluted in 100 µl of serum-free optiMEM and to it 10 µl of FUGENE-6 was added and the complex incubated at RT for 15 min. The medium in the plate was changed and 1.9 ml of fresh medium added to each well. After the 15 min of incubation the DNA-lipid complex was added to each well and the plates incubated overnight with the complex. The medium was replaced next day and the cells were analyzed under the confocal microscope.

Statistical analysis

The data were evaluated by analysis of variance using the InStat program (GraphPad, San Diego, CA). Statistical differences were declared significant at $P < 0.05$ level. Statistically significant data are indicated by asterisks [$P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***)].

Apoptosis assay

MCF 7 cells were plated on confocal dishes on 6/16/08 at 0.5×10^5 cells/dish. The next day the media was changed and cells washed with serum-free opti-MEM prior to transfection. The cells were transfected with 2 µg each of the mock or miR663 plasmids. Transfection was done in 1 ml of serum-free opti-MEM using the Fugene6 transfection agent. After 6 h, 1 ml of complete MCF medium (opti-MEM containing 10% FBS and insulin) was added to each well and left overnight. The next day the media was changed to 2 ml of the complete MCF7 medium. The next day the cells were stained for Alexaflour 663 conjugated- annexinV and counterstained with PI and observed under the confocal microscope. Apoptosis in control cells was induced with 10 µg/ml of Campothecin for 4 h. For staining with Annexin V, the cells were washed with the Annexin binding buffer (10 mM HEPES, pH 7.4 containing 140 mM NaCl and 2.5 mM $CaCl_2$). 25 µl of the Annexin V conjugate was used per 100 µl of buffer and the cells incubated for 15 min at Room temperature. The cells were then washed with the Annexin binding buffer and observed under the confocal microscope. Just prior to the confocal analysis the cells were incubated with 1 µl of PI for 2 min. Images were taken at 10 and 60×.

Immunofluorescence imaging

The cells were seeded onto collagen coated glass chamber slides (Lab-Tek™ II Chamber Slide®, Rochester, NY). At 50–60% confluence, the cells were transfected either with overexpressing miR-663 plasmid pmiRNA1-663 using Fugene 6 transfection reagent. The transfection reaction was performed for up to 24 h and replaced with fresh medium. The cells were observed using a Confocal Laser Scanning Microscope (FV500) (Olympus, Melville, NY, USA).

Luciferase reporter assay

The putative miRNA-663 target site within the 77 bp 3′-UTR region of human AATF mRNA was synthesized with flanking SpeI and HindIII restriction enzyme sites. In addition, the primers with their putative binding site mutated, were

also synthesized from Integrated DNA Technologies as follows, SeedseqAATF forward: GTGCCAAGCTTTAATACACGCAAGGGC**GCTGTCCCGCCCAACCCCGCCT**TTAAACGCCACAAATACTAGTAAAGAGC; SeedseqAATF reverse:GCTCTTTACTAGTATTTGTGGCGTTTAAAGGCGGGGTTGGGCGGGACAGCGCCCTTGCGTGTATTAAAGCTTGGCAC; MutAATF-seedseq-Forward: GTGCCAAGCTTTAATACACGCAAGGGC**GCTGTCCCGCCCAAATGTATAT**TTAAACGCCACAAATACTAGTAAAGAGC; MutAATF-seedseq-Reverse: GCTCTTTACTAGTATTTGTGGCGTTTAAATATACATTTGGGCGGGACAGCGCCCTTGCGTGTATTAAAGCTTGGCAC; MutAATF-Flaseq-Forward: GTGCCAAGCTTTAATACACGCAAGGGC**GCTATAAAGTCCAACCCCGCCT**TTAAACGCCACAAATACTAGTAAAGAGC; MutAATF-Flaseq-Reverse: GCTCTTTACTAGTATTTGTGGCGTTTAAAGGCGGGGTTGGACTTTATAGCGCCCTTGCGTGTATTAAAGCTTGGCAC (blue color within forward primers represents miR-663 putative binding site and respective mutated sites to disrupt binding). The sense and antisense strands of the oligonucleotides were annealed as per our protocol [22]. The annealed primers were digested with SpeI and HindIII and ligated into the multiple cloning site of the pMIRREPORT Luciferase vector (LifeTechnolgies, CA). We transfected cultured HEK293T cells with each of these constructs (pMIR-AATF or pMIR-mutAATF), as well as co-transfecting them with pmiRNA1-663 (pCDH-CMV-MCS-EF1-copGFP) plasmid (System Biosciences, CA) for miR663 overexpression following the transfection protocol as noted above. The protein extract from the transfected cells was collected using RIPA buffer and equal amounts of protein were tested by a Dual Luciferase reporter assay system with Renilla luciferase as control following the manufacturer's instructions.

Results

Distinct miRNAs are upregulated during *P. gingivalis*-epithelial cell interaction

We have previously demonstrated that *P. gingivalis* induce apoptosis of primary human oral epithelial cells [3, 5, 10]. To understand the molecular mechanism of *P. gingivalis* induced apoptosis, we sought to explore the role of miRNAs involved in this process. We stimulated three different donors' primary human oral epithelial cells with heat inactivated and live *P. gingivalis*, ultrapure *E. coli* LPS with or without *Sphingosine-1-phosphate* (S1P) for 24 h. Total RNA was extracted, purified and subjected to miRNA array as described in methods section. The analysis of miRNA arrays uncovered that certain microRNAs are upregulated in live *P. gingivalis* stimulation. Specifically, the upregulated miRNAs included hsa-miR-584, hsa-miR-572, hsa-miR-210, hsa-miR-492, hsa-miR-623, miRPlus_17864, miRPlus_17856 and hsa-miR-663 as seen in Fig. 1a. We also found miR-21 and miR-29a specific for human gingival epithelial cell -12 (HGEC 12) whereas miR-203 was specific for HGEC 9. Further, Principal Component Analysis (PCA) was applied to reduce the dimensionality of our data to capture the variance within the dataset in terms of principle components. The biological replicates in our case with live *P. gingivalis* clustered tightly together while the different treatments were more distant to each other as seen in Fig. 1b. Based on the literature and involvement of miR-663 in cellular apoptosis [43], we aimed to characterize the function of miR-663 in gingival epithelial cells.

MiR-663 induced apoptosis in primary gingival epithelial cells

We sought to validate the microarray findings by real-time PCR for at least two miRNAs. MiR-663 and miR-572 that were overexpressed were selected and real-time PCR was performed. Our data confirmed the upregulated expression of miR-663 during *P. gingivalis* treatment of gingival epithelial cells (Fig. 2a). The data suggested that upregulated miRNA-663 could be part of the apoptosis pathway since we previously demonstrated that *P. gingivalis* induces apoptosis in these epithelial cells [3, 5]. To further evaluate miR-663 function, we transfected epithelial cells with miR-663 by using an overexpression plasmid (miR-663-GFP) and determined its functionality. Our data demonstrated that overexpression of miR-663 induced primary epithelial cell apoptosis (Fig. 2b). Moreover, immunoblot from total protein of miR-663 transfected cells showed the down-regulation of PARP and its cleavage (Fig. 2c).

MiR-663 targets apoptosis antagonizing transcription factor (AATF)

We performed bioinformatic analysis for target prediction using Targetscan from MIT (http://www.targetscan.org) that revealed a strong binding affinity to 3′ untranslated region of AATF mRNA (ENST00000225402.5) (Fig. 3a). To demonstrate the direct binding of miR-663 to AATF, we utilized luciferase reporter assay. The putative miR-663 target site within the 3′-untranslated region of AATF mRNA was biochemically synthesized with *Spe*I and *Hin*dIII restriction enzyme sites as well as oligos with mutated biding sites as described in methods section (detail of sequence and primers and clone confirmation are shown in supplemental file). The annealing, digestion and cloning was done using pMIRREPORTER luciferase system (LifeTechnologies, CA). The plasmid was transfected to HEK293T cells along with miR-663-GFP. Luminescence assays were performed

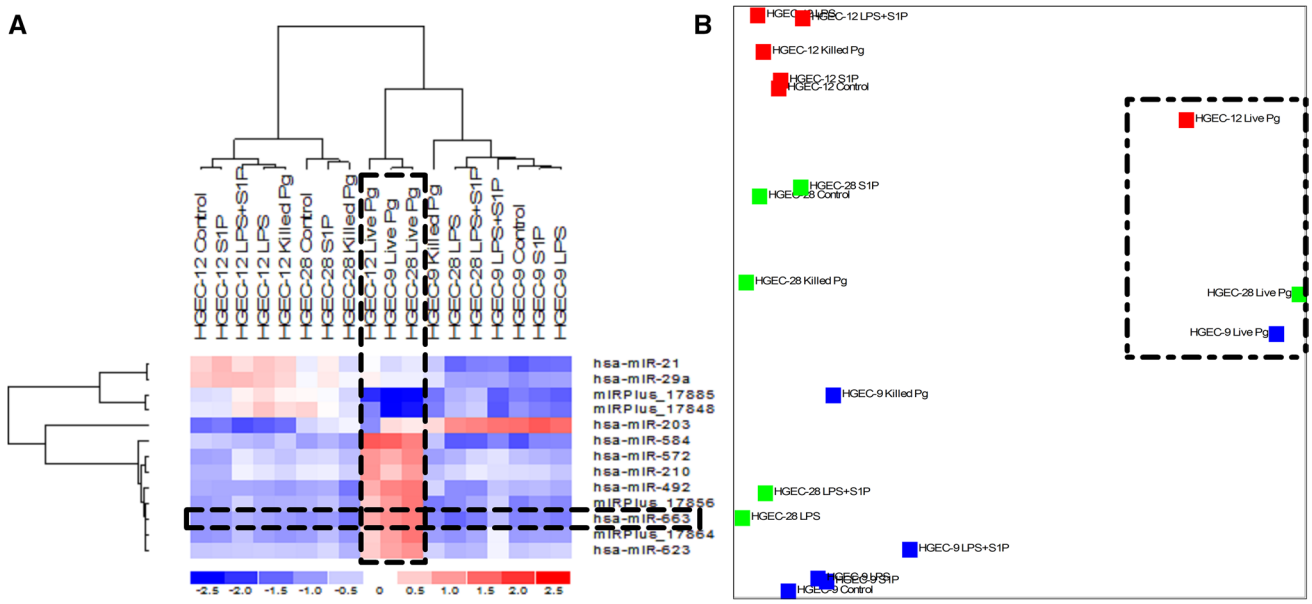

Fig. 1 Differential expression of miRNAs in epithelial cells stimulated with live *P. gingivalis*. Heat Map with unsupervised two-way hierarchical clustering of genes and samples. Each row represents a miRNA and each column represents a sample. The miRNA clustering tree is shown on the left, and the sample clustering tree appears at the top. The color scale shown at the bottom illustrates the relative expression level of a miRNA across all samples: red color represents an expression level above mean, blue color represents expression lower than the mean. Gray color means that the specific miRNA on a given slide has been flagged (i.e., the signal is below background) (**a**). Principal Component Analysis (PCA) analysis using log2(Hy3/Hy5) ratios which passed the filtering criteria on variation across samples; standard deviation < 0.50. HGEC-9 is colored blue, HGEC-12 is red and HGEC is green (**b**)

to determine the luciferase activity. The assay showed significant down regulation of luciferase activity in cells that had the full target region of the putative miR-663 binding site on AATF mRNA (Fig. 3b). To evaluate the efficacy of miR-663 targeting of AATF in epithelial cells, we utilized a protein sample from the overexpression experiment and then immunoblotted it against the AATF antibody. The data demonstrates that the overexpression of miR-663 down-regulated AATF protein confirming the role of miR-663 in inhibiting AATF protein expression (Fig. 3c).

MiR-663 induces MCF-7 cell apoptosis

The unexpected role for miR-663 in inducing epithelial cell apoptosis prompted us to test whether or not it induced apoptosis in cancer cell lines. We utilized the readily available breast cancer cell line MCF-7 to determine the apoptotic function of miR-663. When miR-663-GFP was overexpressed in MCF-7 cell line, significant increase in apoptotic cells were observed by staining for 7AAD followed by immunofluorescence (Fig. 4a). This data suggest that miR-663 not only induces apoptosis in primary oral epithelial cells but is also quite efficient in inducing cell death in the MCF-7 cell line. Moreover, as an additional assay to confirm apoptosis, in a separate experiment the cells were stained for Alexaflour 663 conjugated Annexin V and counterstained with Propidium Iodide (PI). The assay showed significantly increased apoptosis in miR-663 transfected cells (Fig. 4b). Taken together, our data demonstrates that miR-663 is an apoptotic miRNA and that this phenomenon is consistent with the published literature [26, 27, 44, 45].

Discussion

Recent advances using miRNA as a therapeutic agent against certain types of cancers has gained considerable interest. Micro-RNAs are a class of small noncoding RNA molecules ranging from 20 to 23 bp in length and have important gene regulatory functions [22]. Since the discovery of the miRNA, *lin-4* in 1993, over 15,000 miRNAs have been identified and are shown to modulate gene expression [46–48]. Certain miRNAs termed oncomiRs play a causal role in cancer development. Recently, development of cancer has been shown to be directly dependent on a particular oncomiR called miR-155. Blocking this miR-155 led to tumor repression in an animal model [49–51]. Inhibition of oncomiRs using anti-sense oligos in cancer therapies are emerging as novel therapeutics [52]. On the contrary, there are certain miRNAs called "apoptomirs", which are directly involved in the cellular apoptotic process [18]. However, there is very

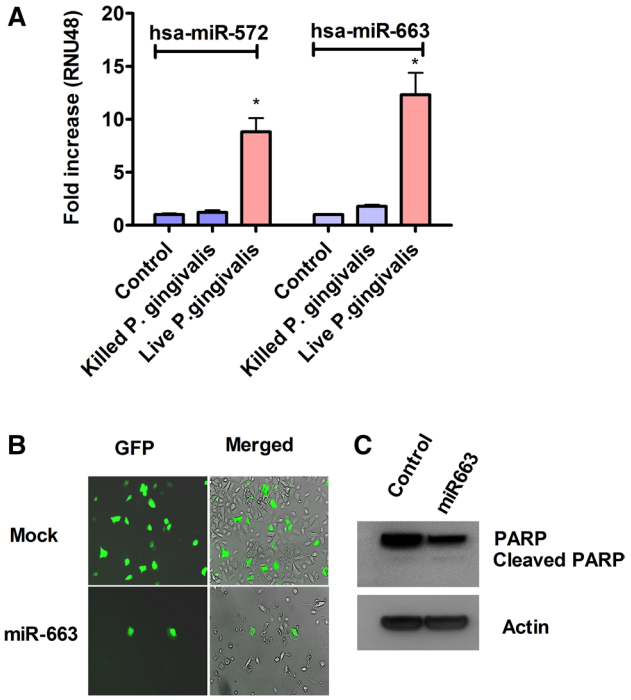

Fig. 2 Validation of microarray data using real-time PCR: real-time PCR data demonstrates the upregulated expression of miR-572 and miR-663 during *P. gingivalis* treatment in gingival epithelial cells. Results are mean ± SEM and asterisks indicated significant difference between control and treatment group ($P < 0.05$) (**a**). MiR-663 induces apoptosis of primary gingival epithelial cells: Primary epithelial cells were transfected with miR-663 overexpression plasmid (miR-663-GFP) and we determined its functionality by fluorescence microscopy. Our data demonstrated that the overexpression of miR-663 induced primary epithelial cell apoptosis as seen by the reduced number of GFP positive cells in miR-663 group when compared to mock transfected group (**b**). MiR-663 transfected samples showed PARP cleavage indicative of cellular apoptosis (**c**)

limited understanding of the potential use of these miRNAs in cancer therapeutics.

In the present study, we identified a target mRNA for miR-663a and assessed its functional role. MiR-663a (miR-663) is present on the chromosome 20 within miR663AHG locus (host-gene) and plays a crucial role in regulating several cellular processes. This miR-663 has been associated with cellular development, viral infections, inflammation and carcinogenesis [43, 44, 53–57]. Ni et al. found that mir-663 was upregulated by oscillatory shear stress in endothelial cells. The authors predicted seven transcription factors namely, FOSB, CEBPB, DDIT3, ATF3, KLF4, BNC1, and MYCN as direct or indirect target for miR-663 [54]. All these transcription factors are known to induce inflammatory responses. Consistent with the above study, the upregulation of miR-663 with Gram −ve *P. gingivalis* treatment in HGECs may be due to innate inflammatory pressure by the activation of NF-kB and other transcription factors with the down regulation of CEBPB. Since *P. gingivalis* is known to induce inflammatory responses and apoptosis of HGECs [3, 5], miR-663's upregulation and participation in the apoptosis pathway, corroborates our findings. Interestingly, miR-663 has been shown to attenuate gastric cancer, prostate cancer and acute lymphoblastic leukemia [56, 57]. On the other hand, down-regulation of miR-663 has also been shown to be associated with lung cancer [45]. Mechanistically, miR-663 has been shown to target JunD gene, elongation factor 1-a, $p21^{(WAF1/CIP1)}$ and heparin sulfate proteoglycan 2, all of which modulate tumor suppressor functions [43, 44]. In this study, we identified a new target for miR-663 namely, AATF. Rb-binding protein AATF is involved in cellular transformation and tumor cell survival, dysregulated autophagy and activity of p53 [58–61]. AATF is upregulated in leukemia cell lines and in patients with chronic lymphocytic leukemia [62]. High levels expression of c-Myc and AATF was reported in a cohort of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) [63]. The expression of AATF disappeared at the time of remission suggesting pro-tumorogenic property for AATF. In addition, silencing of AATF in the primary BCP-ALL cell line boosted chemotherapy [63, 64]. AATF modulates pRb protein's growth suppression process and most importantly its upregulation exerts an anti-apoptotic effect [65–68]. AATF plays a role in metabolism and modulated hypoxia related genes. In the absence of AATF in cells, decreased HIF-1α recruitment was observed to the promoter site under hypoxic conditions suggesting that AATF is necessary for HIF-1α mediated metabolism and cell survival [69]. Functionally, AATF is a nuclear protein that binds to RNA polymerase II and regulates p53, p65 and STAT3 transcription and is involved in cell cycle regulation, DNA damage response and apoptosis [70–72]. Furthermore, Akt1 is a downstream target of AATF that strongly promotes cell survival [72]. Interestingly, in a hepatocellular carcinoma mouse model, a xenograft of human hepatocellular carcinoma cell line QGY-7703 with AATF stably knocked down in a non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mouse model, revealed reduced tumorigenesis and metastases, further confirming anti-tumor activity of AATF [73]. Furthermore, AATF was found to be the key molecule in tumor progression by inhibiting p53 driven apoptosis in the Kras-driven lung adenocarcinoma model [74]. Ectopic expression of mutant AATF in human peripheral blood mononuclear cells (PBMCs) resulted in cellular proliferation [75]. These findings suggest that AATF is a potential target in various types of cancers. Gene silencing of AATF in MCF-7 cells resulted in reduction of both mRNA and protein expression with significant reduction in cellular proliferation along with significant increase in apoptosis [76]. In agreement with this finding, miR-663 overexpression led to HGEC and MCF-7 apoptosis inhibiting AATF mRNA transcription in our experiments, demonstrating the importance of AATF. Sharma [76] further analyzed

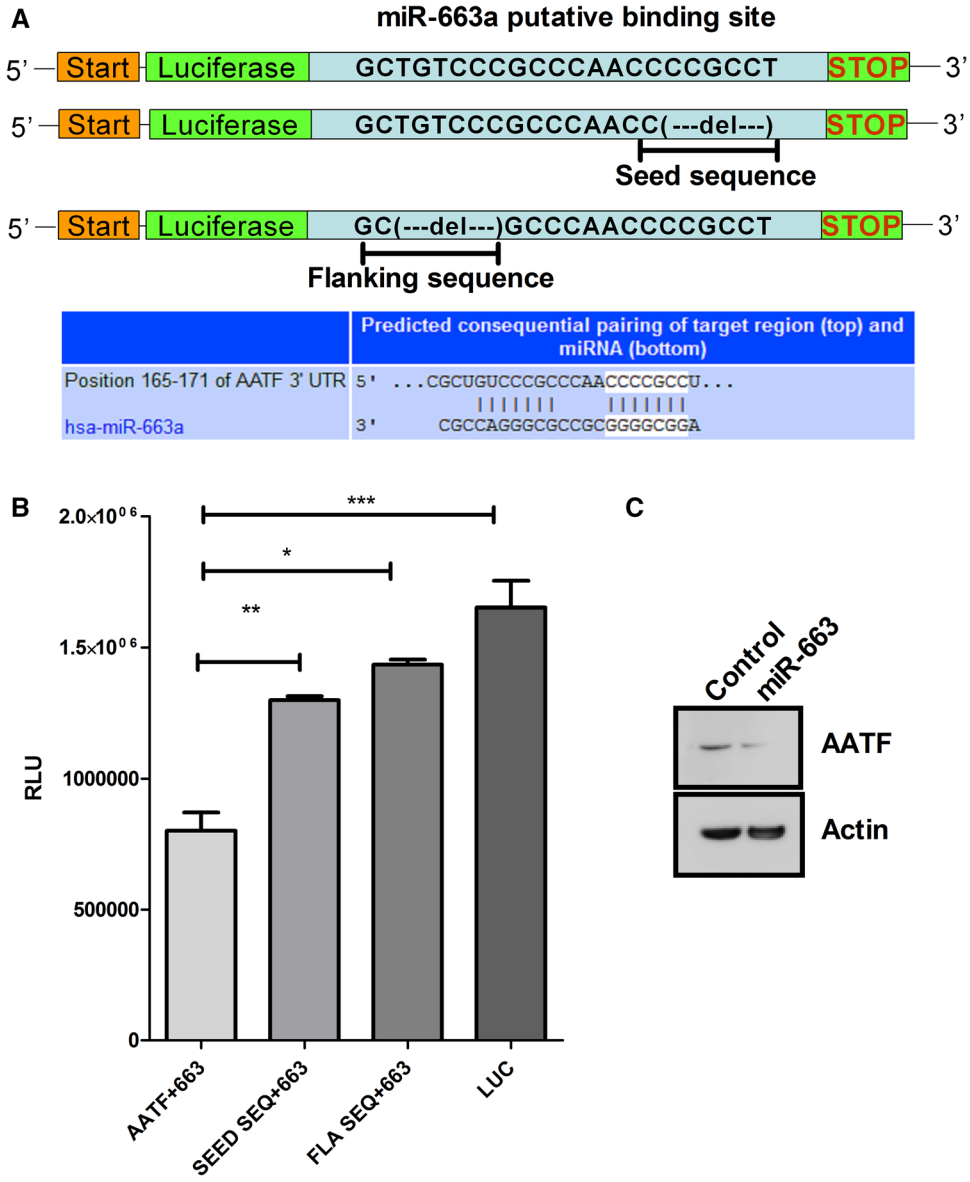

Fig. 3 Confirmation of AATF as a target for miR-663: The putative miR-663 target site within the 3′-untranslated region of AATF as well as oligos with mutated biding sites were transfected to HEK293T cells along with miR-663-GFP (**a**). Luminescence assay showed significant down regulation of luciferase activity in cells that had the full target region of miR-663 binding site on the AATF mRNA (**b**). Immunoblot of miR-663 overexpressed primary epithelial cell protein sample showed down-regulated AATF (**c**)

gene profiling of MCF-7 cells after silencing of AATF and found that apoptosis regulating genes such as Bcl2a1, Birc1, Birc8, TNF, and Pycard, TNF receptor adapter protein were down-regulated. On the contrary, genes for extrinsic cell death pathway such as Fas, Faslg, Fadd and caspases were significantly upregulated. These results suggest that AATF degradation is an essential event in cellular apoptosis. Taken together, modulating the expression level of AATF to limit cellular proliferation and induction of apoptosis may have utility for treating certain cancers. The specific role of miR-663 in targeting AATF suggests both an important biological function in cancer and a potential therapeutic role.

The goal of the present investigation was to understand the role of miR-663 and identify a putative mRNA target. In summary, we have identified *P. gingivalis* induced miRNAs during apoptosis of HGECs, in particular, the role for miR-663. We also identified and demonstrated AATF as a new target for miR-663. These novel findings suggest that miR-663 could be a putative therapeutic agent with AATF as a therapeutic target protein in the management of certain carcinomas.

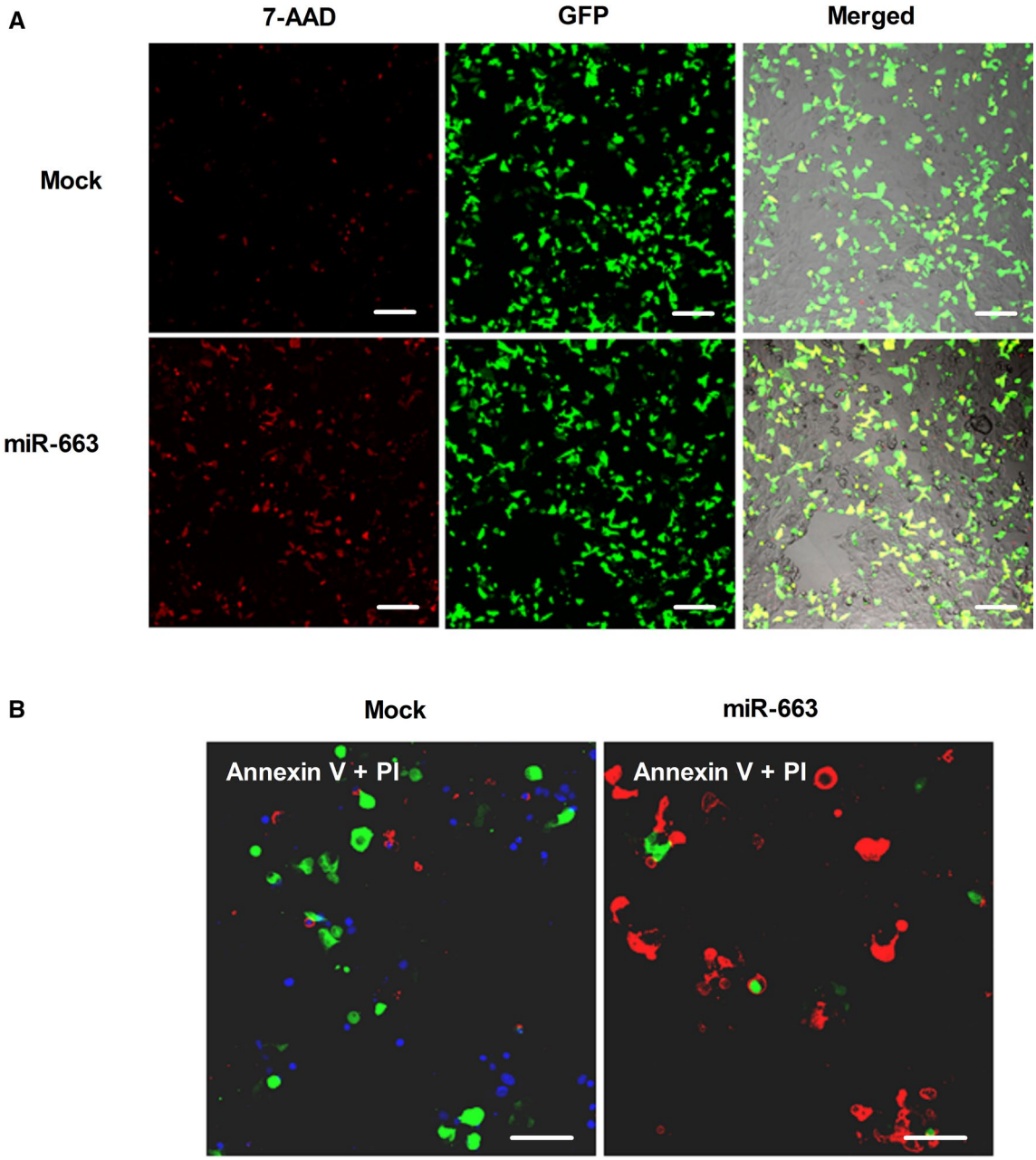

Fig. 4 Mir-663 induces apoptosis of MCF-7 cell line: MiR-663-GFP was overexpressed in the MCF-7 cell line and immunofluorescence was permed by staining with 7AAD. The increased 7-AAD in miR-663 transfected cells suggested that it induces cell death in the MCF-7 cell line (**a**). Further, in a separate experiment the cells were stained for Alexaflour 663 conjugated Annexin V and counterstained with Propidium Iodide (PI). The assay indicates increased apoptosis in miR-663 transfected cells (**b**)

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