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Characterizing methylation regulated miRNA in carcinoma of the human uterine cervix

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ABSTRACT

Gene regulatory mechanisms determine the multistep carcinogenesis process. Two aspects of epigenetics are microRNA (miRNAs) and DNA methylation that regulate distinct biological mechanisms such as metastasis, apoptosis cell proliferation and induction of senescence. Although critical, the interplay between these two epigenetic mechanisms is yet to be completely understood, particularly in cervical cancer. To study the DNA methylation regulation of miRNAs and its potential role in cervical cancer, we investigated the differential methylation pattern of two candidate miRNAs (miR-375 and miR-196a-1) during cervical cancer progression against normal cervical epithelium (NCE) by bisulfite DNA sequencing. miR-375 and miR-196a-1 were hypermethylated in Squamous Cell Carcinoma (SCC) against NCE and Cervical Intra-Epithelial Neoplasia (CIN) ($p < 0.05$). Treatment with demethylating agent reactivated the miR-375 and miR-196a-1 expression in SiHa, HeLa and CaSki cells. *In vitro* artificial methylation by *M.SssI* followed by dual luciferase assay confirmed miR-375 and miR-196a-1 as methylation regulated miRNAs ($P < 0.05$). miR-375 and miR-196a-1 expression levels were negatively correlated with methylation levels in clinical specimens. We further identified Replication Factor C Subunit 3 (*RFC3*) and High Mobility Group AT-Hook 1 (*HMGA1*) as targets of miR-375 and miR-196a-1 respectively by dual luciferase reporter assay. Our analysis indicates that miR-375 and miR-196a-1 are DNA methylation regulated miRNAs whose deregulation may facilitate pathophysiology of cervical cancer.

1. Introduction

Several normal biological and carcinogenesis steps involve epigenetic changes leading to the pathogenesis of human cancers. Two key epigenetic mechanisms such as post-transcriptional RNA regulation by miRNA and control of gene expression by DNA methylation reported to play a key role during tumor progression. microRNAs (miRNAs) are a type of non-coding RNAs (ncRNAs) that are about 22-nucleotides (nts) long, regulating the expression of a gene by acting as post-transcriptional regulators through multiple mechanisms such as mRNA degradation and decay leading to translational repression [1]. miRNA expression is controlled by genetic and epigenetic mechanisms similar to protein-coding transcripts [2,3]. The cross-talk between miRNAs and DNA methylation can occur at several levels. These include a) DNA methylation regulated expression of miRNAs, b) post-transcriptional control of genes by miRNAs which determine DNA methylation, and c) influence of DNA methylation on miRNA biogenesis machinery [3]. Differential methylation of miRNA promoters leading to its aberrant expression has been reported in cervical cancer. For-example, the hypermethylation of miR-124 and hypomethylation of miR-200b promoters is important for cervical carcinogenesis [4,5]. Further, methylation profiling of miRNA promoters and miRNA expression can serve as diagnostic and cancer prognosis indicators [6,7]. For example, the miR-196b hypomethylation was shown to be a potential marker in oral cancer [8]. Wang et al., (2008), also showed that miR-126, miR-143, miR-146a and miR-155 to be substantially deregulated in cervical cancer tissues [9].

Cervical cancer accounts for 569,847 new cases globally, and

Abbreviations: miRNA, microRNA; NCE, Normal Cervical Epithelium; CIN I – III, Cervical intraepithelial neoplasia I – III; SCC, Squamous Cell Carcinoma; UM, Unmethylated; M, Methylated; TF, Transcription Factor; *RFC3*, Replication factor C 3; *HMGA1*, High Mobility Group AT-Hook 1

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Table 1
miRNA selected for bisulfite sequencing, miRNA for promoter activity and miRNA target gene region selected for validation.

miRNA	Co-ordinates (hg19)	Sequence (5′ – 3′)	Number of CpG Sites	Amplicon Size (bp)	Annealing Temperature (°C)	Restriction Site
miR-375	chr2:219,867,576–219,867,955	FP - AAAAAAGATTAGATGTTTTTATTG RP - AAACATAATCCAAAACATCCTAATAA	35	380	57.5	NA
miR-196a-1	chr17: 46,719,038–46,719,347	FP - TAGGTAGGTGGAGTGGTTAAAAT RP - CCAACCCTAAATCTCCTCTC	18	310	60	NA
HMGA1_RT	chr6:34,204,728–34,208,533	FP - GCTCCAAGAAGATCCGCATT RP - GAAGAGTGATGGCTGGGATG	NA	145	60	NA
RFC3_RT	chr13:34,405,433–34,410,259	FP - TGGGAGGTGTATCTGAGGGA RP - ACAGTTCTGAGAGAAGGCCC	NA	148	60	NA
miR-375_Luci	chr2:219,866,795–219,867,944	FP -CGGGGGTACCGCAAATATTGACTCATGGAG RP -ATCCAAGCTTAGAACATCCTGGTGGAGGCGA	NA	1150	60	*KpnI/HindIII*
miR-196a-1_Luci	chr17:46,718,963–46,720,095	FP - GACGGGTACCGTTGCCAGAGAAAAGACGGA RP - TGGAAAGCTTGATCTGCACCTGTGTCCCGG	NA	1133	60	*KpnI/HindIII*
RFC3	chr13: 34,410,866–34,411,517	FP - TGAAGAGCTCTAATTGTATTTTTGTAGGAAA RP -CTAGTCTAGATGAAACATTCAACAATATGGA	NA	652	60	*SacI/XbaI*
HMGA1	chr6: 34,213,037–34,213,892	FP - CCCTTTTAAACTCTGCTCCTTCACTGTTCC RP -GTGGTCTAGACCCCTACACCCTTTATGGTCG	NA	856	60	*DraI/XbaI*

(FP - Forward Primer, RP - Reverse Primer, NA- Not available)

311,365 deaths annually [10]. Reports indicate aberrant oncogenic and tumor-suppressor miRNA expressions play an important role during cervical carcinogenesis. For example, tumor-suppressor miR-183 shown to target MMP-9 which is involved in the extracellular matrix remodeling and hence plays a crucial role in cervical cancer [11]. Similarly, miR-19a/b is an oncogenic miRNA in cervical cancer and is reported to promote cell proliferation and invasion [12] through modulation of key signaling pathways. Previously, we identified several miRNAs that are differentially regulated by DNA promoter methylation during cervical carcinogenesis [5]. These include miR-375 and miR-196a-1 and we chose these based on their expression patterns, locations in and around a CpG island, cognate targets, and potential as biosignatures. The miR-375 whose expression varies among various cancer cell lines is reported to target CTGF thus may alter EGFR signaling [13], PDGF-A preventing invasion and metastasis [14], Her-2 receptor [15], PDK1 [16] and Notch2 [17] thus inhibiting the cell growth from various tumor types. While the miR-196a-1 appears to be co-expressed along with *HOXB9* from the chromosome (chr) 17. Despite the presence of its close homologs 196a-2 at chr12 and miR-196b at chr7, all the three are located within Hox gene clusters, not uniformly expressed among various cancers and may have tissue-specific targets [18]. Taken together, understanding the regulation and biological function of miRNAs at a molecular level is vital in cervical cancer and will facilitate to decipher molecular mechanisms for disease management. The current study aims to investigate the regulation of miR-375 and miR-196a-1, its expression and targets in cervical carcinogenesis.

2. Materials and methods

2.1. Patient sample collection

Participants of the study were volunteers between the age group of 25–75 years (Median age of 50 years) for cervical screening at Kasturba Medical College, Manipal and Mangalore, India. All procedures conducted in research involving human participants were consistent with the ethical norms of the Kasturba Hospital, Manipal ethical committee and also conformed to the protocols in the Declaration of Helsinki of the World Medical Association (WMA) [19]. Informed consents were obtained from every participant prior to the study. The privacy rights of human subjects were always observed. 10 samples for each type (Normal Cervical Epithelium (NCE), Cervical Intraepithelial Neoplasia I - III (CIN I - III) and Squamous Cell Carcinoma (SCC)) were studied. The stages of the tissue biopsy samples were confirmed after being examined histopathologically by pathologists (Table I; Supplementary material).

2.2. Cell line maintenance and 5-aza-2′-deoxycytidine treatment

The maintenance of the cervical cell lines SiHa, CaSki and HeLa were performed in compliance with the guidelines of the American Type Culture Collection (ATCC, USA) (www.atcc.org). While SiHa and HeLa cells were cultured in DMEM containing 10% fetal bovine serum (FBS), CaSki cells were cultured in RPMI media containing 10% FBS (HiMedia, Mumbai, India). For demethylation experiments, cell lines were treated with 10 μM of 5-aza-2′-deoxycytidine (Sigma, USA) for 3 days. As controls, cells without treatment were used. Fresh media containing 5-aza-2′-deoxycytidine was used for the culture and treatment of the cells every day [20]. DNA and RNA were extracted from the cell lines.

2.3. DNA and RNA isolation and purification

Isolation of DNA was performed using a standard phenol-chloroform extraction method [21]. DNA extraction buffer, along with Proteinase K (10 mg/mL) (SISCO Research Laboratory, India) and RNase A (10 mg/mL, Sigma, USA) were added to samples followed by overnight incubation at 37 °C. For RNA isolation from clinical samples, we used the *mir*Vana™ miRNA isolation kit (Thermo Fisher Scientific, USA). The quantity and integrity of the DNA and RNA were assessed on an agarose gel, Qubit fluorometer (Thermo Fisher Scientific, USA) and RNA Nano chip using Bioanalyzer (Agilent Technologies, USA) as per the manufacturer's instructions. The isolated DNA and RNA were stored at −20 °C and − 80 °C until further use.

2.4. Methylation profiling by bisulfite sequencing

Genomic DNA (1.5 μg) was bisulfite converted using the EZ Methylation Kit (Zymo Research, USA) following the respective kits protocol. Details of the primers used in the present study are described in Table 1. The PCR products were purified by gel elution and subjected to direct sequencing using BigDye® Terminator Sequencing Kit in ABI 3130xl Genetic Analyzer (Applied Biosystems, USA), following the manufacturer's instructions. ESME software was used to assess the quality and analyze the level of methylation at each CpG position [22,23].

2.5. Expression profiling by qRT-PCR

miRNA expression was performed using qRT-PCR by ABI 7500 Fast real-time PCR (Applied Biosystems, USA). The level of expression of miR-375 and miR-196a-1 was analyzed in SCC, CIN I - III samples, NCE

samples ($n = 10$) and HeLa, SiHa, and CaSki cervical cancer cell lines. The reverse transcription was performed on total RNA (10 ng/μL) using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) following the manufacturers' protocol. The relative amount of miRNAs [Relative Quantification (RQ)] expression was analyzed using the comparative cycle threshold (CT) method and normalized using RNU6b as endogenous control [24]. Differential expression was estimated using $2^{-\Delta Ct}$ equation, where $\Delta Ct = Ct_{miRNA} - Ct_{RNU6B}$ for tissue samples [25].

2.6. Validation of miR-375 and miR-196a-1 targets by qRT-PCR

MiR-375 (HMGA1_RT) and miR-196a-1 (RFC3_RT) effect on its targets were analyzed by qRTPCR using SyBr Green (Applied Biosystems, USA) method. The reverse transcription was performed from total RNA (1 μg) using High-Capacity cDNA reverse transcription kit (Applied Biosystems, USA). The experiments were repeated three times and performed in triplicates. *β*-actin was used as an endogenous reference gene. The expression level of each miRNA was measured using $2^{-\Delta Ct}$ formula, where $\Delta Ct = Ct_{miRNA} - Ct_{\beta actin}$ as published earlier [25]. Table 1 shows details of the primer sequence, amplicon size, and annealing temperatures.

2.7. Analysis of promoter and 3′-UTR activity

MiR-375 (chr2:219,866,795–219,867,944; 1150 bp) and miR-196a-1 (chr17:46,718,963–46,720,095; 1133 bp) (hg19) promoter regions were amplified using Phusion Taq Polymerase (New England Biolabs, USA), cloned into pGL3-Basic vector (Promega Madison, USA) (Table 1) [21]. The predicted target region of miR-375 [Replication Factor C Subunit 3 (*RFC3)* - chr13: 34,410,866–34,411,517; 652 bp] and miR-196a-1 [High Mobility Group AT-Hook 1 (*HMGA1)* - chr6: 34,213,037–34,213,892; 856b] were cloned into pmirGLO Dual-Luciferase vector (Promega, USA). Sanger sequencing was used to verify the promoter and target constructs. All the experiments were conducted three times independently in duplicates.

2.8. In vitro DNA methylation

SssI methyltransferase (*M.SssI*) (NEB, USA) was used to artificially methylate the promoter region of miR-375 and miR-196a-1 as published earlier [20]. Digestion using methylation insensitive (*Msp*I) and sensitive (*Hpa*II) (NEB, USA) restriction enzymes as well as DNA sequencing (following manufacturer's instruction) of the plasmid DNA was performed to confirm the methylation status before and after treatment with *M.SssI* .

2.9. Transfection and luciferase reporter gene assay

The plasmid constructs (1 μg/well) were transfected into SiHa cells using Lipofectamine LTX reagent (Invitrogen, USA) according to respective kits protocol. Following 48 h of post-transfection, cells were lysed with 1XPassive Lysis Buffer and used for determining the luciferase activity using Dual-Luciferase Reporter Assay System (Promega, USA) in FB12 luminometer (Berthold detection systems, Germany). We used pRL-SV40 as an internal control to normalize and calculate relative luciferase activity [20]. All the experiments were conducted three times independently in duplicates. SiHa cells were co-transfected with 300 ng of pmirGLO constructs and miR-375, miR-196a-1, negative control (NC) mimics (Sigma, USA) at a final concentration of 60 nM for miRNA target detection (NC & *RFC3*, miR-375 & *RFC3*, *RFC3* wild, NC & *HMGA1*, miR-196a-1 & *HMGA1*, *HMGA1*wild and pmiRGLO vector).

2.10. Cell proliferation analysis

SiHa cells were seeded in 96-well plates (1×10^4 cells per well) and 60 nM miRNA or negative control mimics were transfected. The viability of the cells was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay at 24 h, 48 h, and 72 h post-transfection as published earlier [26]. The growth curve was generated on the basis of time and absorbance.

2.11. Computational and statistical analysis

Target prediction of the candidate miRNAs was performed using PITA (score ≤ −10) [27,28], TargetScan (score ≤ −0.45) and miRDB (score ≥ 80) [29,30]. The candidate miRNAs genomic organization was depicted using the UCSC genome browser [31]. We also used SiteSeer, to identify the possible transcription factors [32]. UALCAN was used to analyze and represent the target gene expression status of the miRNA in publically available TCGA datasets [33]; OncoLnc and Circos were used to generate the survival curves and to depicted the predicted miRNA genes [34,35].

R statistical environment [36] and GraphPad Prism (Version 6.0, GraphPad Software, La Jolla California USA, www.graphpad.com) were used to carry out the statistical analyses. ESME software was used for quantitative analysis of methylation at single CpG site resolution. [23]. Percentage of methylation above 20% at individual CpG sites were scored as methylated. MedCalc online software was used to calculate the sensitivity and specificity [37].

3. Results

3.1. miR-375 and miR-196a-1 are differentially methylated between NCE, CIN I - III and SCC

We evaluated the methylation status of miR-375 and miR-196a-1 promoters in NCE, CIN I - III and SCC by bisulfite sequencing. Genomic organization and the region used for analysis is shown in Fig. I Supplementary material. Bisulfite sequencing was performed to identify the differentially methylated CpG sites in the promoter region of miR-375 and miR-196a-1 in NCE, CIN I - III and SCC ($n = 10$) samples. The genomic organization of the validated miRNA, coordinates, and details of the primer are given in Fig. I; Supplementary material and Table 1. The TSS is located 270 bp upstream of the miR-375 and 1080 bp upstream of the miR-196a-1 as predicted by miRStart. *CFAP65* is located upstream and *CRYBA2* is located downstream of miR-375. *HOXB13* is located upstream and *HOXB9* is located downstream of miR-196A-1.

The miR-375 promoter (chr2: 219,867,576–219,867,955) contained 35 CpG sites which spanned the 380 bp region. The CpG sites from 20 to 35 were significantly methylated in SCC against NCE (Fig. 1A) (Table II; Supplementary material). The average methylation across all the 35 CpG sites was found to be 5.2% in NCE, 4.1% in CIN I - III and 20% in SCC samples. The hypermethylation of miR-375 promoter showed 80% sensitivity and 70% specificity to distinguish NCE from SCC (Fig. II; Supplementary material).

The miR-196a-1 promoter (chr17: 46,719,038–46,719,347) contained 18 CpG sites which spanned the 310 bp region. The CpG sites from 3 to 11 and 14–18 were significantly methylated in SCC against NCE (Fig. 1B) (Table II; Supplementary material). The average methylation across all the 18 CpG sites was found to be 13% in NCE, 17.5% in CIN I - III and 37% in SCC. The hypermethylation of miR-196a-1 promoter showed 80% and 100% sensitivity and specificity respectively, between SCC and NCE samples (Fig. II; Supplementary material).

Promoter regions of miR-375 and miR-196a-1 were methylated in SiHa, CaSki and HeLa cells. We treated SiHa, CaSki, and HeLa cell lines with the demethylating agent and performed bisulfite sequencing to test the effect of demethylation on specific CpG sites. Our data showed that the DNA methylation decreased in specific CpG sites in the treated (5-aza-2′-deoxycytidine) cervical cancer cell lines in contrast to the untreated cells (Fig. 2).

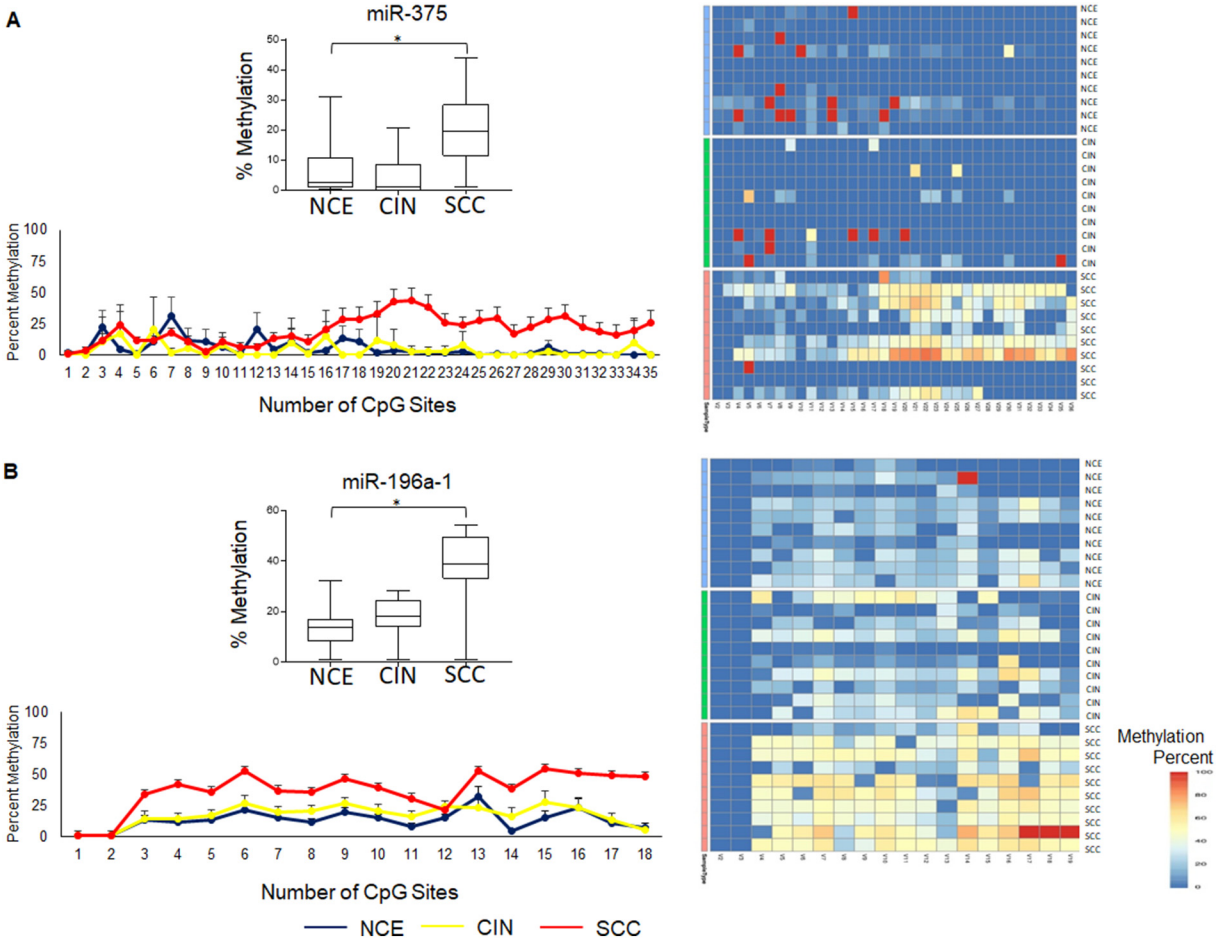

Fig. 1. Consolidated methylation analysis of miRNA associated probes. The purified PCR product was sequenced using Automated DNA Sequencer and was read using Chromas and Bio-Edit software. A) miR-375 and B) miR-196a-1. The methylation analysis of two differentially methylated miRNAs was identified in our study. The methylation analysis was performed in 10 each of NCE, CIN I - III, and SCC samples by bisulfite sequencing. The box plot represents methylation level averaged over all CpG sites analyzed for each category of samples. The line graph represents methylation levels at individual CpG sites where the samples are colour coded, where NCE is a red line, CIN I - III is a yellow line and SCC is a red line. X-axis representing the number of CpG sites in the amplicon sequenced and the Y-axis representing the percent methylation of the individual sites, along with the respective error bars. Heat map of four differentially methylated genes in 10 each of NCE, CIN I - III, and SCC samples. Samples arranged as NCE, CIN I -III, and SCC (horizontal); and CpG sites (vertical). The frequency of methylation is represented by colors with blue and red representing 0%–100% methylation. The heat map was drawn using R package (CRAN) of pheatmap tool. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

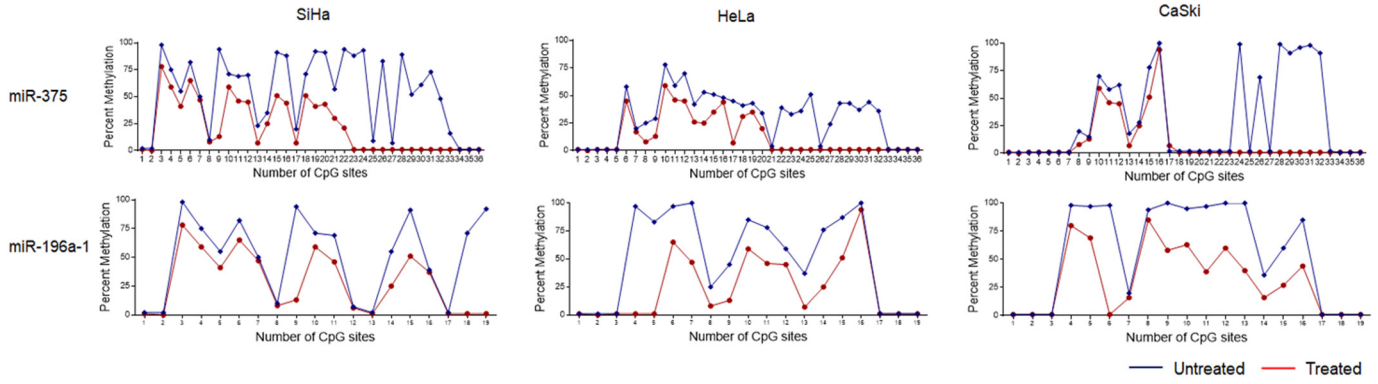

Fig. 2. Candidate miRNAs validated by bisulfite sequencing in SiHa, HeLa, and CaSki. Cells were treated with 10 µM 5-aza-2′-deoxycytidine for 5 days and demethylated CpG sites. The line graphs represent the methylation status of individual CpG sites before and after treatment with 5-aza-2′-deoxycytidine. Our results suggested the demethylation of multiple CpG sites in all the three cell lines tested. The untreated and treated group is represented in blue and red colors respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

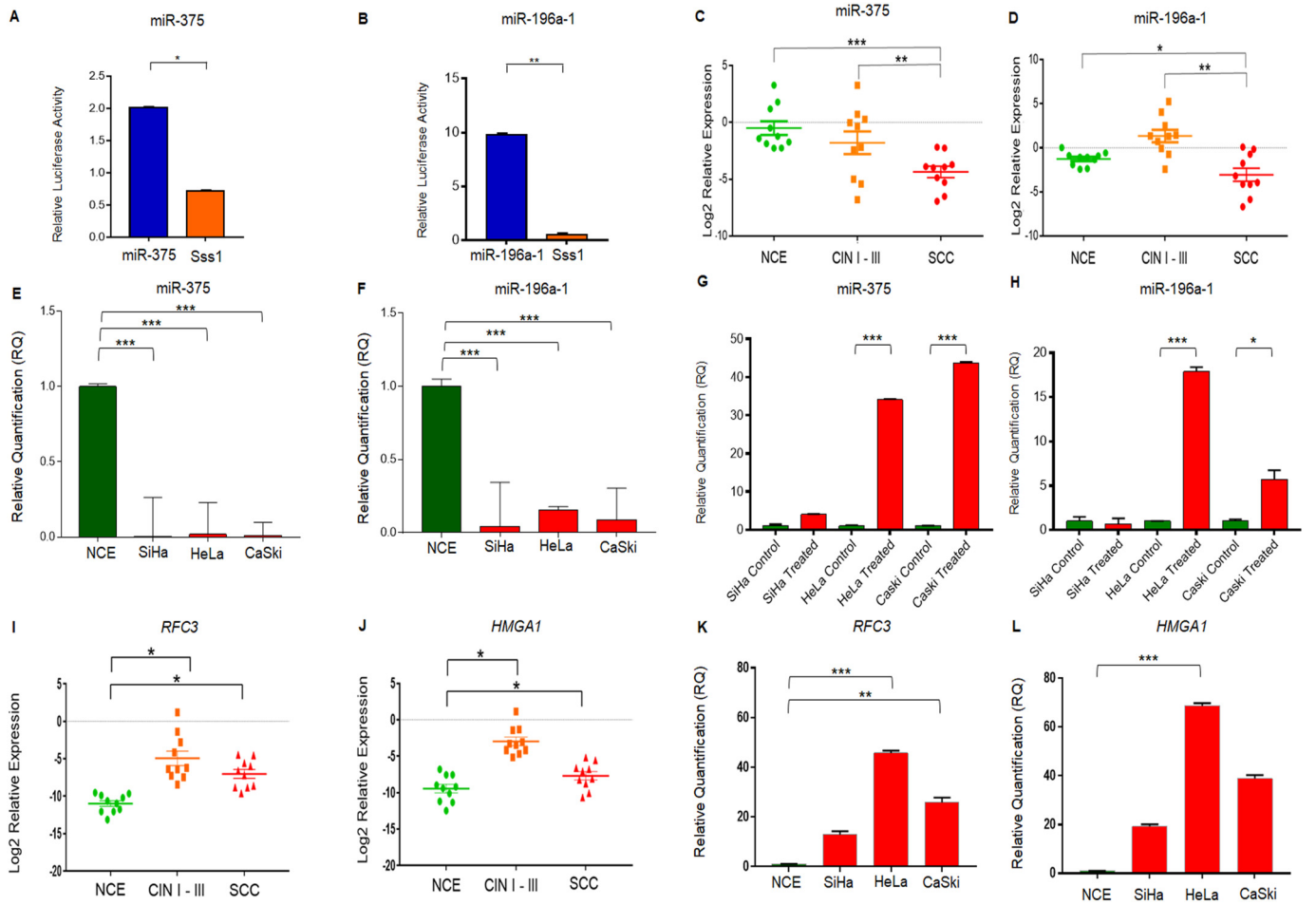

Fig. 3. Characterization of gene expression at the transcriptional level of the (A) miR-375 and (B) miR-196a-1 by transient transfection experiment in pGL3 basic vector. miRNA expression of (C) and (D) miR-375 and miR-196a-1 has shown to be downregulated in SCC samples. (E) and (F) Relative quantitative expression (RQ) profile by real time-qPCR of miR-375 and miR-196a-1 in cell lines. (G) and (H) Relative quantitative expression (RQ) profile by real time-qPCR of the miR-375 and miR-196a-1. The data were normalized by using the expression of RNU6b as an endogenous control. Green and red bars represent control and treated cell lines (SiHa, HeLa, and CaSki respectively). (I) and (J) *RFC3* and *HMGA1* expression levels are identified as upregulated in SCC to NCE. miRNA expression of (K) and (L) *RFC3* and *HMGA1* has shown to be upregulated in cervical cell lines compared NCE. (* p-value < 0.05, **p-value < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Promoter activity by transient transfection assay

The promoters of miR-375 and miR-196a-1 were characterized by transient transfection and luciferase reporter assay. Our results indicate that the predicted promoters regions namely miR-375 and miR-196a-1 showed robust promoter activity for miR-375 (chr2:219,866,795–219,867,944; 1150 bp) and miR-196a-1 (chr17:46,718,963–46,720,095; 1133 bp) (hg19). Further, artificial methylation significantly diminished the promoter activity of the miR-375 and miR-196a-1 (Fig. 3A and B). The luciferase activity of *M.SssI* treated constructs were decreased by 1.3 fold in miR-375 and 9-fold in miR-196a-1 relative to the unmodified constructs in SiHa cells ($p < 0.05$). Our data established that miR-375 and miR-196a-1 are methylation regulated miRNAs in cervical cancer (Fig. 3A and B). Potential transcriptional factor binding sites search using SiteSeer tool revealed *TERT* and *POLB* has the potential to bind to miR-375; and *EGFR* and *IL6* to miR-196a-1 respectively (Fig. III; Supplementary material).

3.3. MiR-375 and miR-196a-1 are downregulated in SCC and cervical cancer cell lines

MiR-375 and miR-196a-1 expression were assessed by qRT-PCR in NCE, CIN I - III, SCC tissues ($n = 10$) and cervical cancer cell lines. Significantly lower expression of miR-375 ($p < 0.0001$) (Fig. 3C) and miR-196a-1 ($p < 0.05$) (Fig. 3D) was observed in SCC when compared against NCE ($p < 0.01$). miR-375 and miR-196a-1 were also significantly lower in CaSki, HeLa and SiHa cell lines (Fig. 3E and F). Treatment with 10 µM of 5-aza-2′-deoxycytidine enhanced miR-375 and miR-196a-1 expression when compared to the untreated cervical cancer cells (CaSki, HeLa and SiHa). We identified miR-375 (3, 34- and 43-fold increase in SiHa, HeLa, and CaSki treated cells, $p < 0.05$) and miR196a-1 (17 and 5-fold increase in HeLa and CaSki treated cells, $p < 0.0001$) expression as significantly increased upon treatment with 5-aza-2′-deoxycytidine when compared to untreated cells (Fig. 3G and H). These results suggested that miR-375 and miR-196a-1 are methylation regulated miRNAs in cervical cancer.

3.4. MiR-375 and miR-196a-1 targets RFC3 and HMGA1 in cervical cancer

We have used miRDB (score ≥ 80) and PITA (score ≤ −10) tools and identified miR-375 and miR-196a-1, to target 56 and 255 protein-coding genes (Fig. IV; Supplementary material). Further, we selected *RFC3* and *HMGA1* as one of the targets for miR-375 and miR-196a-1 respectively. These targets were found to be near to the poly(A) tail

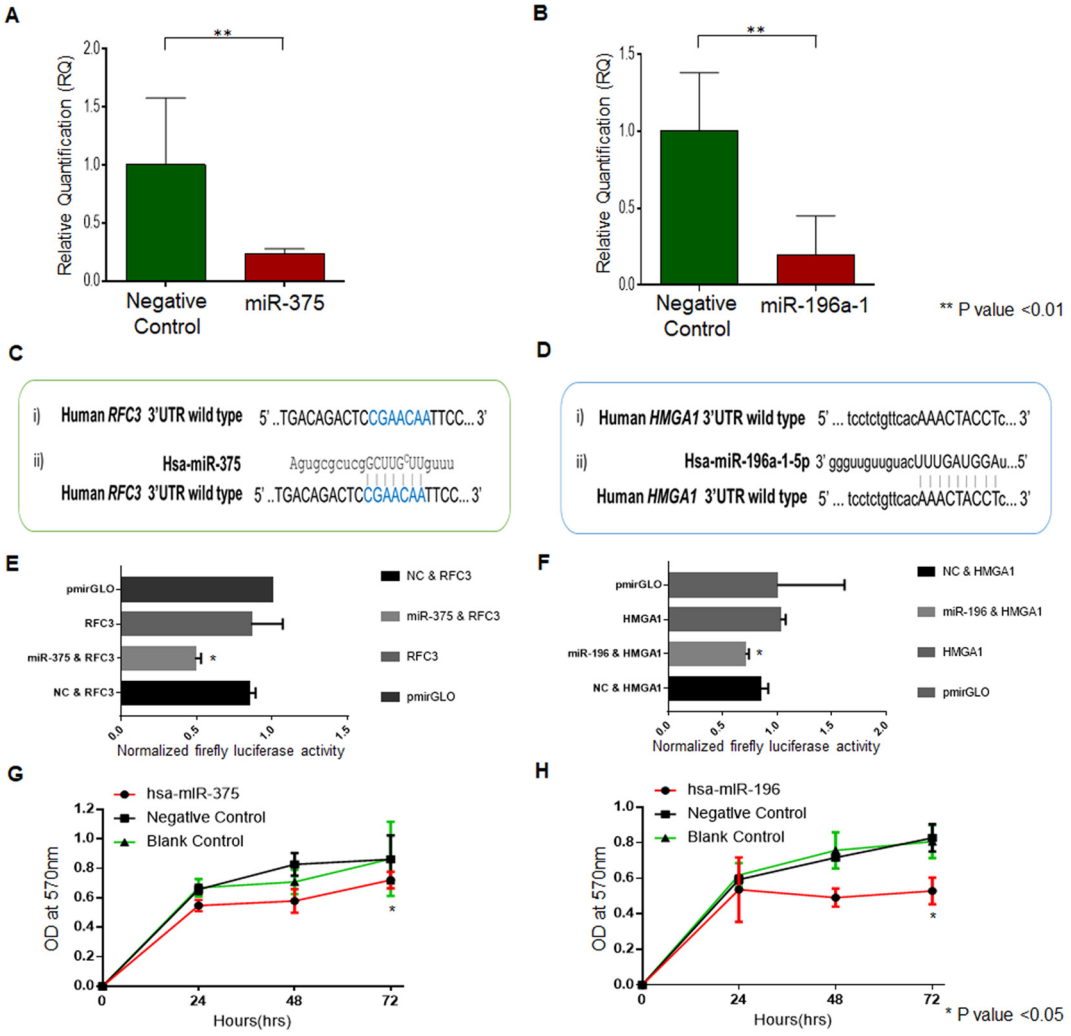

Fig. 4. Real-time PCR analysis indicated that miR-375 and miR-196a-1 downregulated the expression of *HIPK3* (E) and *RBBP6* (F) respectively in comparison with the negative control. Statistical analysis was performed using a *t*-test and p-value < 0.05 was considered to be statistically significant. (C and D) The miR-375 and miR-196a-1 binding sites in the RFC3 and HMGA1 3′-UTR. The miR-375 and miR-196a-1 mimics showed significant suppression of luciferase activity of the vector containing (E) RFC3 and (F) HMGA1 binding sites ($p < 0.05$). All the experiments were performed in duplicates and repeated thrice. $P < 0.05$ were considered as statistically significant. The growth curve showed a significant decrease in cell proliferation rate upon transfection with miR-375 (G) and miR-196a-1 (H) ($p < 0.05$). Our results suggest that miR-375 and miR-196a-1 are inhibitors of SiHa cell proliferation.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which is more susceptible for effective targeting [38]. We have tested the expression of *RFC3* and *HMGA1* in NCE, CIN I - III, SCC ($n = 10$ each) and cervical cancer cell lines by qRT-PCR. We have identified significantly higher expression of *RFC3* and *HMGA1* in SCC and CIN compared to NCE with concomitant downregulation of their respective miRNAs (p-value < 0.05) (Fig. 3I and J). *RFC3* and *HMGA1* showed increased expression in CaSki, HeLa, and SiHa cell lines (Fig. 3K and L). Further, the cervical cancer TCGA expression data (CESC) of *RFC3* and *HMGA1* also showed upregulation in successive stages of SCC compared to normal samples (Table III and Fig. V; Supplementary material). Taken together, our data suggest that miR-375 and miR-196a-1 are methylation regulated miRNAs which represses transcription of *RFC3* and *HMGA1* in cervical cancer.

3.5. RFC3and HMGA1 is a direct target of miR-375 and miR196a-1

miR-375 and miR-196a-1 repressed *RFC3* and *HMGA1* activity respectively compared to the negative control (Fig. 4A and B). Significant suppression of the luciferase activities of the constructs containing *RFC3* (chr13: 34,410,866–34,411,517) and *HMGA1* binding sites (chr6: 34,213,037–34,213,892) (hg19) ($p < 0.05$) was shown by the miR-375 and miR-196a-1 mimics. We showed that *RFC3* and *HMGA1* expression was inhibited by miR-375 and miR-196a-1 respectively, through direct binding to their respective 3′-UTR region (Fig. 4E and F).

3.6. Forced expression of miR-375 and miR-196a-1 suppresses proliferation of SiHa cells

Downregulation of miR-375 and miR-196a-1 in SCC samples and cervical cancer cell lines, suggests a possible tumor-suppressor role of these miRNAs in cervical cancer. In order to understand the biological function of miR-375 and miR-196a-1 in cervical cancer, we performed cell proliferation assay. SiHa cells were transfected with miR-375, miR-196a-1 and negative control mimics. The assessment of the growth curve showed a significant reduction in the proliferation of SiHa cells. Our findings suggest that miR-375 and miR-196a-1 are potential proliferation inhibitors of SiHa cells (Fig. 4G and H).

4. Discussion

Epigenetic alterations are one of the key events associated with the origin and development of cancer [39]. The miRNA expression and subsequent biological effects are often deregulated in cancer *via* multiple mechanisms including aberrant promoter DNA methylation and can be used as a potential marker with diagnostic significance [40]. Hence, identification and understanding of miRNA dysregulation during cervical cancer is of clinical relevance and there are limited studies on cervical cancer. This study was carried out to find the effect of differential methylation on the expression of miRNA, miR-375 and miR-196a-1 and their targets in cervical cancer. Our study demonstrated that the promoters of miR-375 and miR-196a-1 are hypermethylated in SCC leading to its downregulation at transcript levels.

Our *in silico* analyses have shown the regulated target of miR-375 in cervical cancer has shown to be involved in PI3K AKT signaling pathway (*EGFR, JAK2, LAMC2, BCL2, IGF1R, IL2RA,* and *THBS1*), Hippo signaling pathway (*YAP1, FRMD6, TGFB2, SMAD7, SNAI2*) and TGF beta signaling pathway (*CDKN2B, SMAD7, TGFB2,* and *THBS1*). For miR-196a-1, MAPK signaling pathway (*RASGRP1, COL1A1, COL1A2, ITGAV,* and *CCND2*), and PI3K AKT signaling pathway (*PDGFRA, COL1A1,* and *COL1A2*) are some of the regulated targets of miR-196a-1 in cervical cancer as predicted by miRNACancerMap [41] (Table IV; Supplementary material).

The impact of DNA methylation on the expression of miRNA was assessed in miR-375 and miR196a-1 by (i) RT-PCR after the induction of demethylation (ii) bisulfite sequencing to identify the CpG methylation of a specific region of the promoter distinct to the miRNA. It was observed that upon treatment with 5-aza-2′-deoxycytidine, miR-375 and miR-196a-1 the level of methylation decreased in the individual CpG sites compared to the untreated cell lines. In addition, by *in vitro* methylation by using *M.SssI* showed decrease promoter methylation of miR-375 and miR-196a-1 suggesting DNA methylation directly contributes to miRNA gene silencing.

The miR-375 was first discovered as a pancreas-specific miRNA that regulates insulin secretion and is reported to play a role in carcinogenesis [42]. miR-375 is reported to targets multiple oncogenes AEG-1, YAP1, IGF1R, and PDK1 and is downregulated in many cancers [43]. Wang et al., 2011 showed that decreased expression of miR-375 hinders the invasion and migration of cells by targeting SP1 transcription factor in cervical cancer [44]. In our study, we identified the CpG density of miR-375 of 35 sites spanning a 380 bp region and methylation density changes were observed in the CpG sites 20–35. The average methylation percent was found to be 5.2% in NCE, 4.1% in CIN I - III and 20% in SCC. The promoter methylation status and the miRNA expression validation by RT-PCR showed a direct association. miR-375 was identified as hypermethylated and coincided with its reduced expression levels in SCC samples. By treatment of miR-375 promoter region with *M.SssI* we observed a significant reduction in luciferase reporter plasmids activity. This could suggest that DNA methylation in the CpG island containing promoter regions inhibits transcription activity.

Hou et al., 2014 showed that poor survival was associated with upregulation of miR-196a in cervical cancer patients. miR-196a targets FOXO1 and p27Kip1 to induce cell cycle progression and proliferation through enhancing G1/S phase transition [45]. Liu et al.,2015 demonstrated that HPV 16 E5 downregulates the miR-196a leading to the transformation of normal cervical cells to carcinoma cells [46]. We identified miR-196a-1 related to CpG shores and tested miRNA's regulation and its biological effects. miR-196a-1 containing 18 CpG sites spanning 310 bp showed significant hypermethylation of CpG sites 3–11 and 13–18. The overall percentage of methylation was 13% in NCE, 17.5% in CIN and 37% in SCC. miR-196a-1 qPCR expression showed an inverse correlation with its promoter methylation status. By treatment with of miR-196a-1 promoter with *M.SssI* we identified a substantial reduction in the luciferase reporter plasmids activity indicating that DNA methylation impedes the transcription activity driven from the CpG island containing promoter regions.

Translational repression by miR-375 and miR-196a-1 was demonstrated by using miRNA mimics using dual luciferase assays. Our studies are consistent with the published reports, stating that methylation of the miRNA promoter region, miRNA expression and the target genes of the miRNA show an inverse correlation validating the general hypothesis that silencing induced by methylation affects the downstream gene expression in cervical cancer, [47]. We also observed by dual-luciferase reporter assay that miR-375 and miR-196a-1 directly bind to the 3′-UTR region of *RFC3* and *HMGA1* respectively. miR-375 and miR-196a-1 as direct targets of *RFC3* and *HMGA1* respectively.

Target prediction analyses showed *RFC3* and *HMGA1* as targets of miR-375 and miR-196a-1, respectively. Previous reports have shown that *RFC3* is a component of the BRCA1-associated genome surveillance complex that serves as an abnormal DNA structures sensor [48]. Also, stabilizes stalled replication fork in response to DNA damage and stimulating pathways such as recombination-dependent DNA replication [49]. Upregulation of *RFC3* expression has been reported in ovarian, esophageal, liver and breast cancers [50–53] suggesting an oncogenic role. The triple negative breast cancer progression was promoted by the elevated levels of *RFC3 via* EMT-pathway [53].

HMGA1 is a bonafide chromatin protein shown to be proto-oncogene. The level of HMGA proteins has reduced substantially in normal differentiated somatic cells [54,55] However, in cancers, levels of HMGA proteins are generally high, with poor patient prognosis [56–58]. This association is so coherent and common that many distinct kinds of human cancer use high concentrations of HMGA proteins as clinical diagnostic markers [59–64] We confirmed in this study that *RFC3* and *HMGA1* are direct targets of miR-375 and miR-196a-1 respectively. Further investigations on the roles of *RFC3* and *HMGA1* in cervical cancer are warranted.

5. Conclusion

In summary, our results demonstrated that miR-375 and miR-196a-1 are aberrantly methylated and expressed in cervical cancer. Although, the differential methylation of the specific CpG sites showed very high sensitivity and specificity requires further detailed investigation. The methylation patterns could serve as a useful biomarker for risk assessment or tumor diagnosis or as a clinically important parameter that could be exploited as a potential target for therapy. Further studies in cervical cancer are needed to explore the biological function of these miRNAs.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Author contributions

Kapaettu Satyamoorthy - Conceptualization; Vinay Koshy Varghese and Vaibhav Shukla - Data curation; Vinay Koshy Varghese, Vaibhav

Shukla - Formal analysis; Kapaettu Satyamoorthy - Funding acquisition; Vinay Koshy Varghese, Vaibhav Shukla - Investigation; Vinay Koshy Varghese, Vaibhav Shukla, Jishnu Padacherri Vethil - Methodology; Shama Prasada Kabekkodu, Kapaettu Satyamoorthy - Project administration; Deeksha Pandey, Krishna Sharan - Clinical samples, histopathology reports, clinical co-relation analysis; Shama Prasada Kabekkodu, Kapaettu Satyamoorthy - Supervision; Vinay Koshy Varghese, Vaibhav Shukla, Jishnu Padacherri Vethil - Validation; Vinay Koshy Varghese, Vaibhav Shukla - Visualization; Vinay Koshy Varghese, Vaibhav Shukla, Shama Prasada Kabekkodu - Roles/Writing - original draft; Vinay Koshy Varghese, Vaibhav Shukla, Jishnu Padacherri Vethil, Shama Prasada Kabekkodu, Kapaettu Satyamoorthy Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116668.

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