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Enumeration of deregulated miRNAs in liquid and tissue biopsies of cervical cancer

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HIGHLIGHTS

- Established miRNA signatures in cervical cancer serum and tissue specimens
- miRNAs from exonic regions were downregulated in cervical cancer specimens.
- Key miRNA clusters were identified, and they show similar patterns of expression.
- miR-409-3p and miR-454-3p targets *MTF2* and *ST18* respectively.
- miR-17, miR-32, miR-454 and miR-409 may be suitable as biosignature using serum specimens

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ABSTRACT

Objective. The altered miRNAs expression in cervical cancer tissue can be a critical player during tumorigenesis, may contribute to tumor cell heterogeneity and may determine distinct phenotypes within the tumor. Recent studies have highlighted the role of circulating miRNAs as a minimally-invasive biomarker and its potential as biosignature to complement routine tissue-based procedures.

Methods. In order to determine whether miRNAs in serum can indicate changes in cervical tissue specimens, we performed small RNA sequencing and selected miRNAs were validated using qRT-PCR in serum and tissue specimens ($n = 115$). Further, luciferase assay were performed to investigate the interactions between hsa-miR-409-3p and hsa-miR-454-3p binding sites on 3′UTR region of *MTF2* and *ST18* respectively.

Results. We have identified a total of 14 differentially expressed miRNAs common in serum and tissue specimens. Among them, hsa-miR-17-5p, hsa-miR-32-5p and hsa-miR-454-3p were upregulated while, hsa-miR-409-3p was downregulated in serum and tissue of cervical cancer subjects. Our *in-silico* small RNA sequencing data analysis identified isomiRs and classified miRNA into clusters and subtypes (exonic, intronic and intergenic) with respect to the expression status in serum and tissue specimens. Expression level of hsa-miR-409-3p and hsa-miR-454-3p were inversely correlated with their target genes *MTF2* and *ST18* levels respectively in human cervical cancer specimens. Luciferase assay demonstrated that hsa-miR-409-3p and hsa-miR-454-3p functionally interacts with 3′-UTR of *MTF2* and *ST18* respectively to decrease their activity.

Conclusion. Our results support the significant role of circulating miRNAs in disease dissemination and their potential utility as biosignatures of clinical relevance.

1. Introduction

Among women, cervical cancer accounts for the third most common cancer worldwide with >500,000 new cases reported annually. In India, approximately 96,922 new cases and 60,078 mortality are reported in

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the year 2012 [1]. Pap smear test and human papillomavirus (HPV) typing have reduced the incidence of cervical cancer worldwide [2]. Testing for HPV has high sensitivity but lacks specificity when compared to cytology, while cytology has limited sensitivity (38% to 65%) [3]. The omic-based discovery approach has helped to identify various proteins, specific RNA species and genomic aberrations such as mutations, in/dels, copy number variations among others relevant to diseases. The discovery of miRNAs as a regulator of gene expression has resulted in the extensive investigation to determine the role of miRNAs in the development and progression of various pathophysiological conditions including cancer. Identification of differentially expressed miRNAs from tissue specimens has provided impetus to cancer biomarker studies for diagnosis, prognosis and better clinical management [4].

Since their discovery in 1993, small non-coding RNAs termed miRNAs, reported to play an essential role in gene regulation by acting at the post-transcription level. Other attributes of miRNAs include a) complimentary interactions with other miRNAs to restrict transcription process [5], b) formation of triple helix with double-stranded DNA at sequence-specific sites to alter gene expression [6], c) interfere with miRNA mediated gene expression through the formation of RNA G-quadruplex structures in mature miRNAs [7], and d) modification of miRNA by m6A RNA methylation to add additional layer of complexity [8]. In addition, miRNAs may cause various pathophysiological conditions including cancer. Xie et al. have reported up-regulation of hsa-miR-205 in cervical cancer tissues [9] and elevated levels of hsa-miR-205 in serum represents an independent prognostic biomarker for cervical cancers [10]. Although studies have been performed to profile global miRNA expression in cervical tissues, results are limited from other sources such as extracellular fluids (plasma, urine or serum) and exfoliative cytology tissues. The circulatory miRNAs can be stable, resistant to extreme pH and repeated freeze and thaw cycles making it as a suitable candidate for clinical application. Use of circulating miRNA profiles can also enhance utility of the standard tissue biopsy biomarkers. This has been demonstrated in several cancers such as breast and lung cancer [11]. Taken together, studies suggest that miRNA profiling from serum can be useful as minimally invasive method for diverse clinical application [12]. However, such studies are limited in cervical cancer.

In this study, we have investigated the miRNA expression in normal cervical epithelium (NCE), normal healthy control (NHC), cervical intraepithelial neoplasia (CINI-III) and squamous cell carcinoma (SCC) tissue and serum specimens by performing small RNA sequencing and validated the expression of hsa-miR-454, hsa-miR-32 and hsa-miR-409 and hsa-miR-17 in an independent panel of serum and tissue specimens.

2. Material and methods

2.1. Specimens collection and cell line maintenance

Histpathologically confirmed NCE, CIN(I-III) and SCC tissue biopsies and blood samples were collected from participants who visited Kasturba Medical College, Manipal and Mangalore, Karnataka, India for cervical screening after obtaining ethical clearance from Kasturba Hospital ethical committe, MAHE (Table S1A). All the functional studies were performed using SiHa, Hela and CaSki cells (NCCS, Pune) that were maintained according to ATCC guidelines (American Type culture collection, www.atcc.org).

2.2. RNA extraction from primary tissues and serum specimens

Total RNA was extracted from serum and tissue specimens using *mir*Vana™ miRNA isolation kit (Ambion® Life technologies, USA) and miRNeasy Serum/Plasma kit (Qiagen, Germany) respectively. The specimens were stored at −80 °C until further use. The quality and quantity of RNA were assessed by agarose gel electrophoresis, RNA 6000 Nano chip in 2100 Bioanalyzer instrument (Agilent Technologies, USA), and Qubit Fluorometer (Thermo Fisher Scientific, USA).

2.3. Library construction and small RNA sequencing

The small RNA sequencing was performed in NCE, CIN(I-III) and SCC tissue specimens and, NHC and SCC serum specimens ($n = 15$ each). In brief, total RNA from the specimens was enriched for small RNAs using Total RNA-Seq Kit v2 (Thermo Fisher Scientific, USA). The quality and quantity of samples enriched for small RNA were analyzed using the Agilent® small RNA kit in the 2100 Bioanalyzer instrument. Barcoded samples were used for library construction using Total RNA-Seq Kit v2. Amplification of the templates was performed using Ion OneTouch™ 2 system and Ion OneTouch™ ES (Thermo Fisher Scientific, USA). The enriched product was loaded onto a P1™ chip and run on an Ion Proton system™ (Thermo Fisher Scientific, USA).

2.4. Reverse transcription and real-time PCR quantitation

cDNA was synthesized from total RNA using TaqMan microRNA assay protocol (Thermo Fisher Scientific, USA). RNU6b and miR-16 were used as endogenous control for tissues and serum specimens respectively. The relative expression level of each miRNA in individual specimens was calculated as published earlier [13]. Expression of *MTF2* and *ST18* were determined using SyBr green method (Applied Biosystem, USA). High-Capacity cDNA reverse transcription kit (Applied Biosystem, USA) was used to synthesize cDNA. β-Actin was used as endogenous control. Real-time PCR was performed using ABI 7500-fast real-time PCR (Applied Biosystem, USA). Primer sequences were given in table S1B. All experiments were performed in triplicates.

2.5. Construction and transfection of plasmid

The 3′-UTR regions of human *MTF2* and *ST18* were amplified from normal fibroblast human genomic DNA and cloned into pmirGLO Dual-Luciferase miRNA target expression vector (Promega, USA). DNA sequencing by Sanger's method and restriction digestion was performed to verify the sequence identity and orientation. SiHa cells were grown to about 60% confluence in a 12 well tissue culture plate and were co-transfected with 300 ng of pmirGLO constructs along with 60 nM miRNA and negative control mimics using lipofectamine LTX reagent (Thermo Fisher Scientific, USA). After 48 h post-transfection, cells were lysed with 1× lysis buffer. Firefly and Renilla activities were measured using Dual-Luciferase Reporter Kit (Promega, USA) in an FB12 Luminometer (Berthold Technologies, Germany). The experiments were conducted in duplicates and repeated three times.

2.6. Cell proliferation assay

SiHa (1×10^4) were plated in 96-well plated for overnight and transfected on the next day with 60 nm of miRNA mimics and negative control. Cell proliferation was identified at 24, 48, and 72 h post transfection using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-terazolium bromide (MTT). Absorbance was measured at 570 nm and 630 nm. The experiment was repeated three times.

2.7. Computational and statistical analysis

CAP-miRSeq pipeline was used for read pre-processing, alignment, distribution of RNA types, and differential miRNA expression analysis between NCE, NHC and SCC specimens [14]. LogFC (log2fold change) >1 and <−1 were assigned to up- and down-regulated miRNAs with FDR (False Discovery Rate) <0.05 [15]. Principal component analysis (PCA) was performed using ClustVis [16]. Targets were predicted using miRDB (Score ≥ 80) [17], and DIANA microT V4 (Score ≥ 0.9) [18]. Pathway analysis was performed using DAVID 6.8 tools [19].

IsomiR quantification was performed using miraligner from SeqBuster software [20].

3. Results

3.1. Small RNA sequencing

High-throughput sequencing produced 3,312,020, 6,043,334 and 5,517,882 reads from NCE, CIN(I-III) and SCC tissue specimens, respectively and 534,337 and 499,870 reads from NHC and SCC serum specimens respectively. Reads aligned to the human genome (GRCh38) were 1,716,547 (51.82%), 3,321,778 (54.96%) and 2,227,036 (40.36%) from NCE and SCC tissue specimens while 210,180 (39.33%) and 344,612 (68.94%) were from NHC and SCC serum specimens respectively. The total numbers of unique small RNA reads were 1,281,033, 2,398,825 and 1,796,380 in tissue specimens, from NCE, CIN(I-III) and SCC respectively and 44,897 and 254,435 in serum specimens, from NHC and SCC respectively (Fig. 1A). After alignment RNAs were classified into different categories such as known miRNAs, lncRNAs, protein coding and others (tRNAs, rRNAs, snRNAs). Any RNA product which is not defined by known RNA features was annotated as misc_RNA. There was a total of 10%, 2% and 3% of misc_RNA reads from NCE, CIN and SCC tissue specimens respectively. Interestingly, 88% and 46% reads were from NHC and SCC serum specimens were of misc_RNA types respectively (Fig. 1B). To distinguish unique miRNA expression data from NGS for cervical cancer tissue and serum specimens from their healthy counterparts, we performed principal component analysis (PCA) and demonstrated that miRNA expression profiling can distinguish normal from cancer specimens both in tissues and serum specimens (Fig. 2B).

3.2. IsomiR identification

To describe the variability in miRNA biogenesis, isomiR analysis was performed on small RNA sequence data. We have selected ≥10 read counts for our downstream analysis. In total, we have identified 352 unique miRNAs with isomiRs. The most common modifications of isomiRs were with 3′-trimming in both tissue and serum specimens. Almost 76% (266/352) of miRNAs showed more than one modification (Fig. 2A).

3.3. Identification of differentially expressed miRNAs

We compared the differential expressed miRNA based on three criteria: 1) present only in serum, 2) present only in tissue and 3) present both in tissue and serum. A volcano plot-based filtering was performed to identify the significant differentially expressed miRNAs (Fig. 2C). In total, we have identified 59 upregulated and 74 downregulated miRNAs in SCC against NCE, whereas there were 44 upregulated and 58 downregulated miRNAs in SCC, against CIN (logFC > 1 or logFC<−1 with FDR < 0.05). There were no miRNAs with significant differential expression in CIN when compared to NCE (FDR > 0.05). Similarly, we have identified a total of 18 upregulated and 15 downregulated miRNAs (logFC > 1 or logFC < −1 with FDR < 0.05) in SCC compared to NHC serum specimens (Fig. 3).

Our data identified 16 miRNA clusters, of which 7 were upregulated and 9 were downregulated in SCC compared with NCE (Fig. 4A). However, we identified the expression of 19 miRNAs on chromosome (chr) 14 miRNA cluster (hsa-miR-381-3p, hsa-miR-411-5p, hsa-miR-154-5p, hsa-miR-299-5p, hsa-miR-655-3p, hsa-miR-656-3p, hsa-miR-369-5p, hsa-miR-376c-3p, hsa-miR-379-5p, hsa-miR-654-3p, hsa-miR-487b-3p, hsa-miR-382-5p, hsa-miR-409-5p, hsa-miR-134-5p, hsa-miR-494-3p, hsa-miR-485-3p, hsa-miR-495-3p, hsa-miR-376b-3p and hsa-miR-543) which were downregulated in SCC specimens. In serum specimens, we have identified one overexpressed miRNA cluster from chr13 (mir-17/19b-1) in SCC compared to NHC. Taken together, these results suggest that the changes in miRNA clusters expression may be associated with cervical carcinogenesis.

The 133 differentially expressed miRNAs of SCC when compared to NCE indicated that 47 were intronic, 6 were exonic and 80 were intergenic. Out of 47 intronic miRNAs, 25 were upregulated (logFC>1 and FDR < 0.05) and 22 were downregulated (logFC<−1 and FDR < 0.05). All of 6 exonic miRNAs were downregulated (logFC<−1 and FDR < 0.05). Among the 80 intergenic miRNAs, 34 were upregulated (logFC>1 and FDR < 0.05) and 46 were downregulated (logFC<−1 and FDR < 0.05). When 102 differentially expressed miRNAs of SCC were compared against CINI-III, 33 were found to be intronic, 5 were exonic and 64 were intergenic. Out of 33 intronic miRNAs, 22 were upregulated (logFC>1 and FDR < 0.05) and 11 were downregulated (logFC<−1 and FDR < 0.05) in SCC compared to CINI-III. All the 5 exonic miRNAs were found to be downregulated (logFC<−1 and FDR < 0.05) in SCC compared to CINI-III. Besides, out of 64 intergenic miRNAs, 22 were upregulated (logFC>1 and FDR < 0.05) and 42 were downregulated (logFC<−1

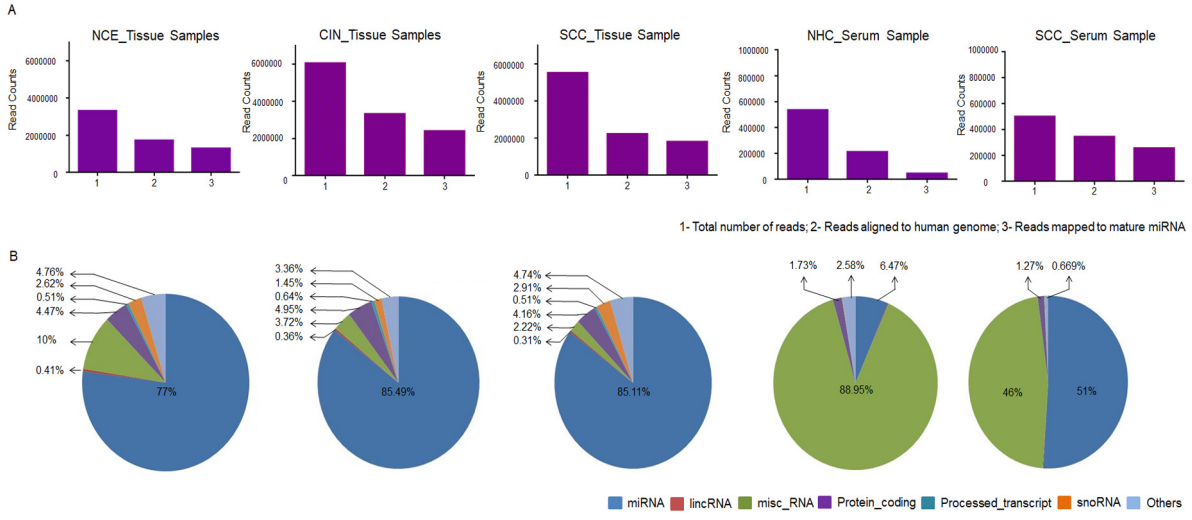

Fig. 1. A) Read abundance obtained by small RNA sequencing from cervical tissues and serum. Sequencing reads combined from 15 specimens of NCE, CIN and SCC of tissue and NHC and SCC serum specimens were mapped to human genome (GRCh38) and represented. B) Pie chart depicting the percentage of reads from combined 15 specimens of tissue and serum to detect the distribution of all RNAs such as miRNAs, lncRNAs, protein coding and pseudogenes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

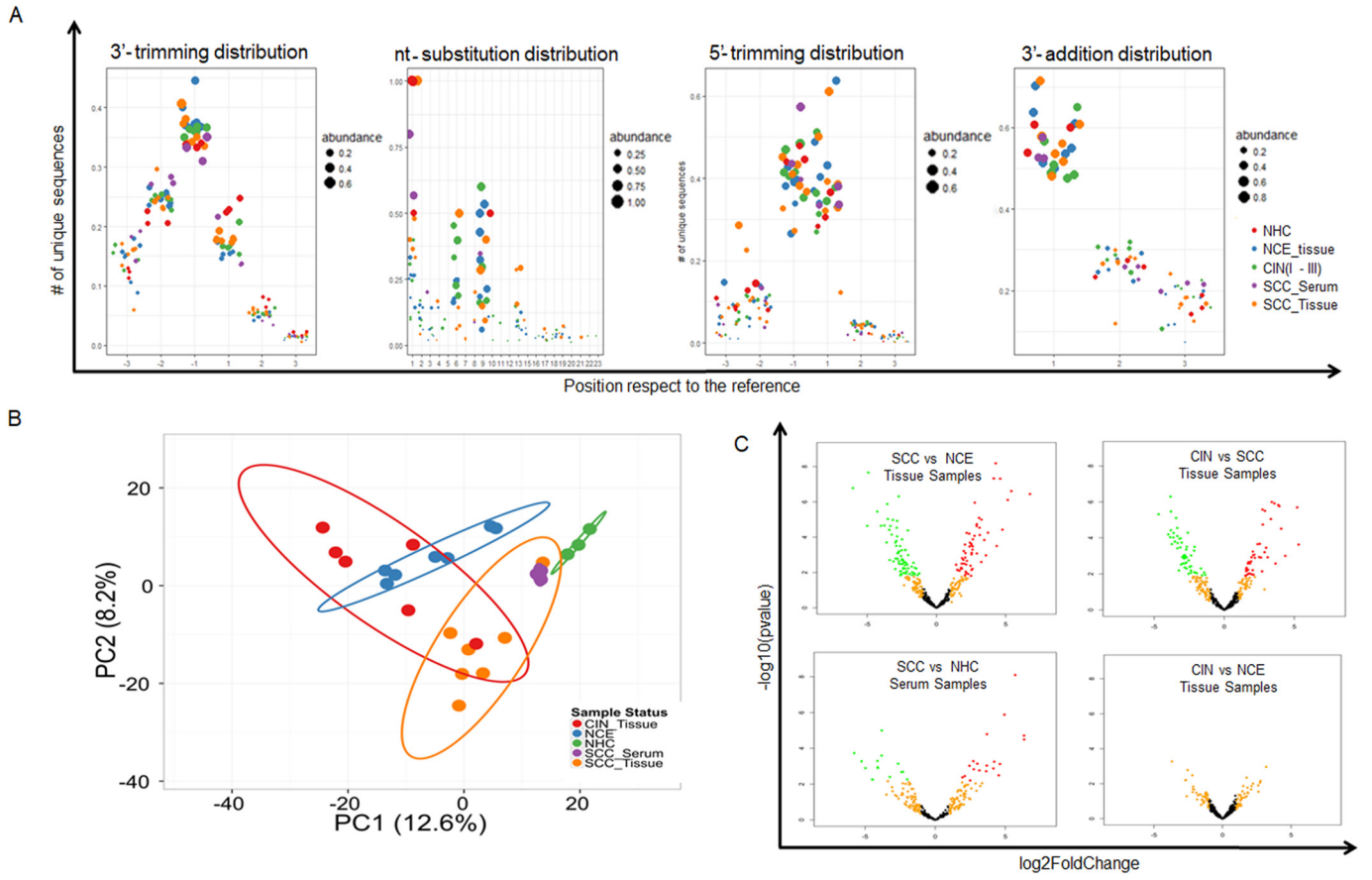

Fig. 2. A) IsomiR distribution in NCE, CIN(I-III), and SCC tissue and NHC and SCC serum specimens (nt-nucleotides). B) Principal Component analysis-X axis show principal component 1 and Y axis shows principal component 2 that explain 12.6% and 8.2% of the total variance, respectively. Normalized reads were used for principal component analysis. Ellipses and shapes show clustering of the specimens. C) Volcano plot analysis of differential expressed miRNAs in two datasets. The expression differences in miRNAs between SCC against NCE, SCC against CIN(I-III) and CIN(I-III) *vs* NCE tissue specimens and NHC against SCC serum specimens is plotted on the X axis, and False Discovery Rate (FDR) significance is plotted on the Y axis ($-\log_{10}$ scale). The black dots represent no significant change, orange dots represent logFC >1 and < −1 and red dots represent logFC of >1 and FDR < 0.05 and green represent logFC < −1 and FDR <0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and FDR < 0.05) in SCC compared to CINI-III. Further, out of 33 differentially expressed miRNAs in SCC against NHC, 12, 1 and 20 were found to be intronic, exonic and intergenic respectively wherein, 6 intronic miRNAs were shown to be upregulated (logFC>1 and FDR < 0.05) and 6 downregulated (logFC<−1 and FDR < 0.05). A single exonic miRNA was found to be downregulated. Out of 20 intergenic miRNAs, 8 were upregulated and 12 were downregulated in SCC against NHC (Fig. 4B).

Further, we compared the differentially expressed miRNAs (logFC>1 or <−1 with FDR < 0.05) in tissue and serum specimens. We have identified a total of 14 common miRNAs between tissue and serum specimens (Table S2). Out of 14, 12 were shown to be upregulated (hsa-miR-20b-5p, hsa-miR-451a, hsa-miR-15b-3p, hsa-miR-486-5p, hsa-miR-20a-5p,hsa-miR-18a-5p, hsa-mir-17-5p, hsa-miR-15a-5p, hsa-miR-32-5p, hsa-miR-223-3p, hsa-miR-454-3p and hsa-miR-182-5p) and 2 were downregulated (hsa-miR-409-3p and hsa-miR-483-5p) in serum and tissue SCC specimens compared to healthy controls. A total 119 and 19 miRNAs were unique in tissue and serum specimens respectively. Heat map analysis was generated to compare the expression of common miRNAs identified both in tissue and serum specimens. The analysis has shown the similar trend of expression in SCC tissue and serum specimens compared to the healthy control counterparts (Fig. 4C).

3.4. Validation of miRNA expression and their target gene by qRT- PCR

The validation of miRNAs [3 upregulated (hsa-miR-17-5p, hsa-miR-32-5p and hsa-miR-454-3p) and 1 downregulated (hsa-miR-409-3p)] was performed by qRT-PCR in 10 each of tissues (NCE, SCC) and serum (NHC, SCC) specimens respectively. We have selected these miRNAs based on our *in-silico* analysis which showed (i) expression of hsa-miR-17-5p, hsa-miR-32-5p, and hsa-miR-454-3p as upregulated; while, hsa-miR-409-3p as downregulated both in tissue and serum SCC specimens compared to their healthy controls and ii) targets multiple oncogenic pathways such as MAPK, PI3K-AKT and many more (Table S3). The expression of miRNA was also performed in cervical cell lines. The results from qRT-PCR were in agreement with our small RNA sequencing analysis result (Fig. 5).

Further, we selected one upregulated (miR-454-3p) and one downregulated (miR-409-3p) miRNAs based on their expression in SCC as compared to normal counterparts. *MTF2* and *ST18* were identified to contain an hsa-miR-409-3p and hsa-miR-454-3p binding site respectively. The expression of *MTF2* and *ST18* were analyzed in cervical cancer tissue specimens and cervical cancer cell lines by qRT-PCR. The expression of *ST18* was downregulated (Fig. 6A and B) and *MTF2* was upregulated (Fig. 6C and D) in cervical cancer tissue specimens as opposed to normal specimens. The Expression of *MTF2* and *ST18* were in inverse correlation to their respective miRNA expression.

3.5. Hsa-miR-409-3p and hsa-miR-454-3p down regulates MTF2 and ST18 respectively by directly binding to it's 3′-UTR

The dual luciferase reporter assay showed the upregulation of hsa-miR-454-3p and hsa-miR-409-3p significantly inhibited the relative luciferase activity of *ST18* (Fig. 6G) and *MTF2* (Fig. 6I) 3′-UTR in SiHa cells

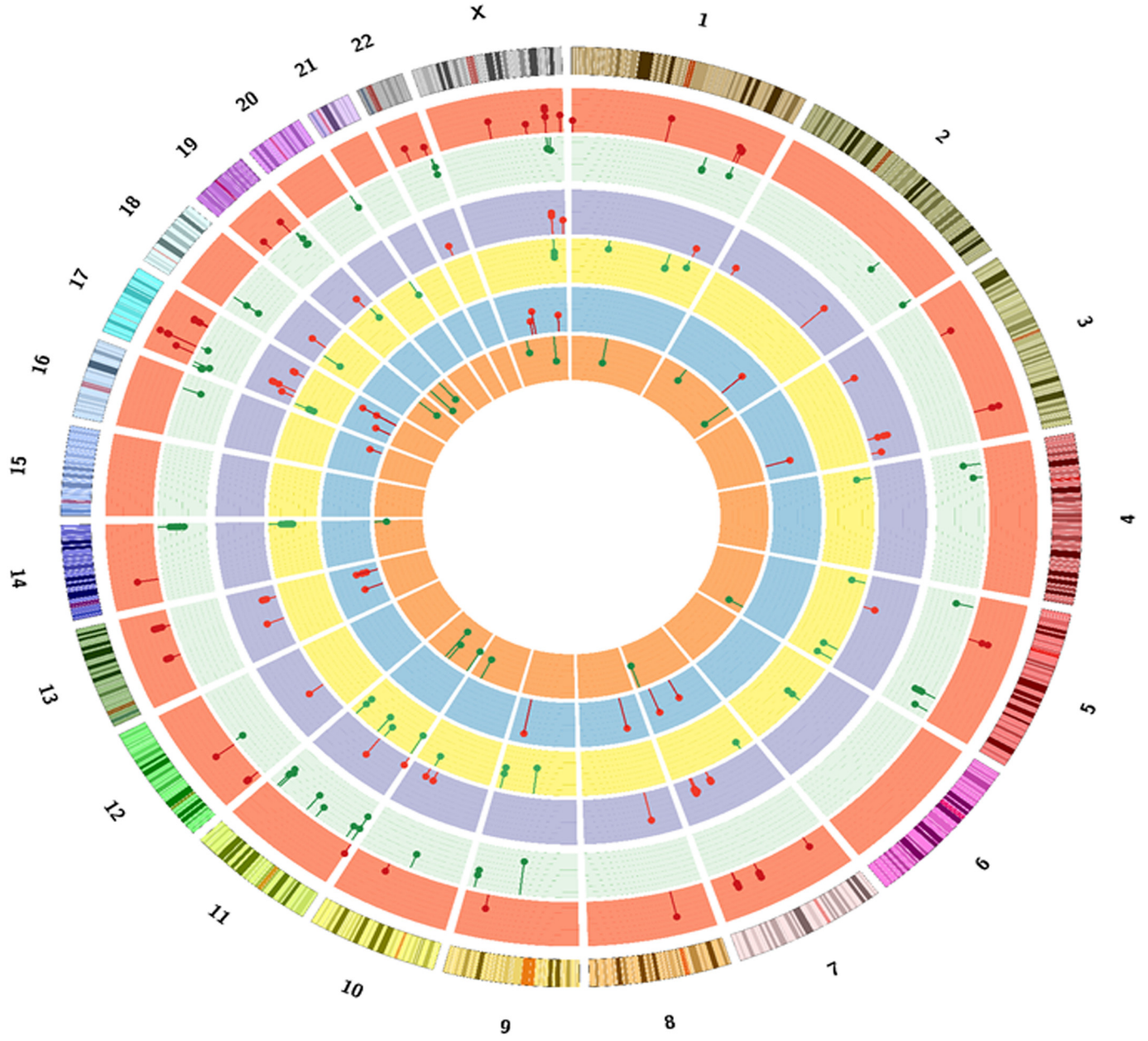

Fig. 3. Genomic distribution of differential expressed miRNAs. Circos showing human genome summarizing over expressed (log2FC > 1 and FDR < 0.05) and downregulated (log2FC < −1 and FDR < 0.05) miRNAs in SCC relative to NCE, SCC relative to CINI-III and SCC relative to NHC. The outermost tracks (red and green) represent miRNA in SCC relative to NCE, middle track (purple and yellow) represents miRNAs in SCC relative to CINI-III and innermost track (orange and blue) represents SCC relative to NHC. (Red colour-over expressed, green colour-downregulated). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respectively. In addition, qPCR analysis demonstrated that the overexpression of hsa-miR-454-3p and hsa-miR-409-3p substantially reduced the expression of *ST18* (Fig. 6H) and *MTF2* (Fig. 6J) and respectively in SiHa cells. These findings indicate that *MTF2* and *ST18* expression were inhibited by hsa-miR-409-3p and hsa-miR-454-3p directly bindings to 3′-UTR of its transcripts.

3.6. Knockout of miR-454-3p and forced expression of miR-409-3p inhibited SiHa cell proliferation

SiHa cell were transfected with hsa-miR-409-3p, negative control mimics, anti-miR-454-3p and anti-negative control mimics. The proliferation curve showed a significant decrease in cell viability. Thus, our results suggest that hsa-miR-454-3p can activate (Fig. 6K) and hsa-miR-409-3p can suppress the cell proliferation (Fig. 6L).

4. Discussion

Although cervical cancer is preventable, it is still prevalent in many parts of the world and common in developing countries. Persistent HPV infections, lifestyle coupled with genetic and epigenetic changes are proposed as major cause of cervical cancer. Studies have demonstrated that the deregulated miRNA expression in tissues may play an important role in the pathophysiology of cervical cancer and may serve as a potential biomarker [21]. Minimally-invasive strategies are being increasingly used to show the utility of miRNA profiling from biological fluids to understand the mechanism of tumor progression and as an alternative and sensitive method for tissue biopsy based invasive procedure. The miRNAs are shown to be released into the circulation by active cell secretion or by necrotic and apoptotic cells and they exist either freely, in exosomes or associated with Argonaut or HDL proteins [22]. Thus, miRNA profiling using serum and tissue

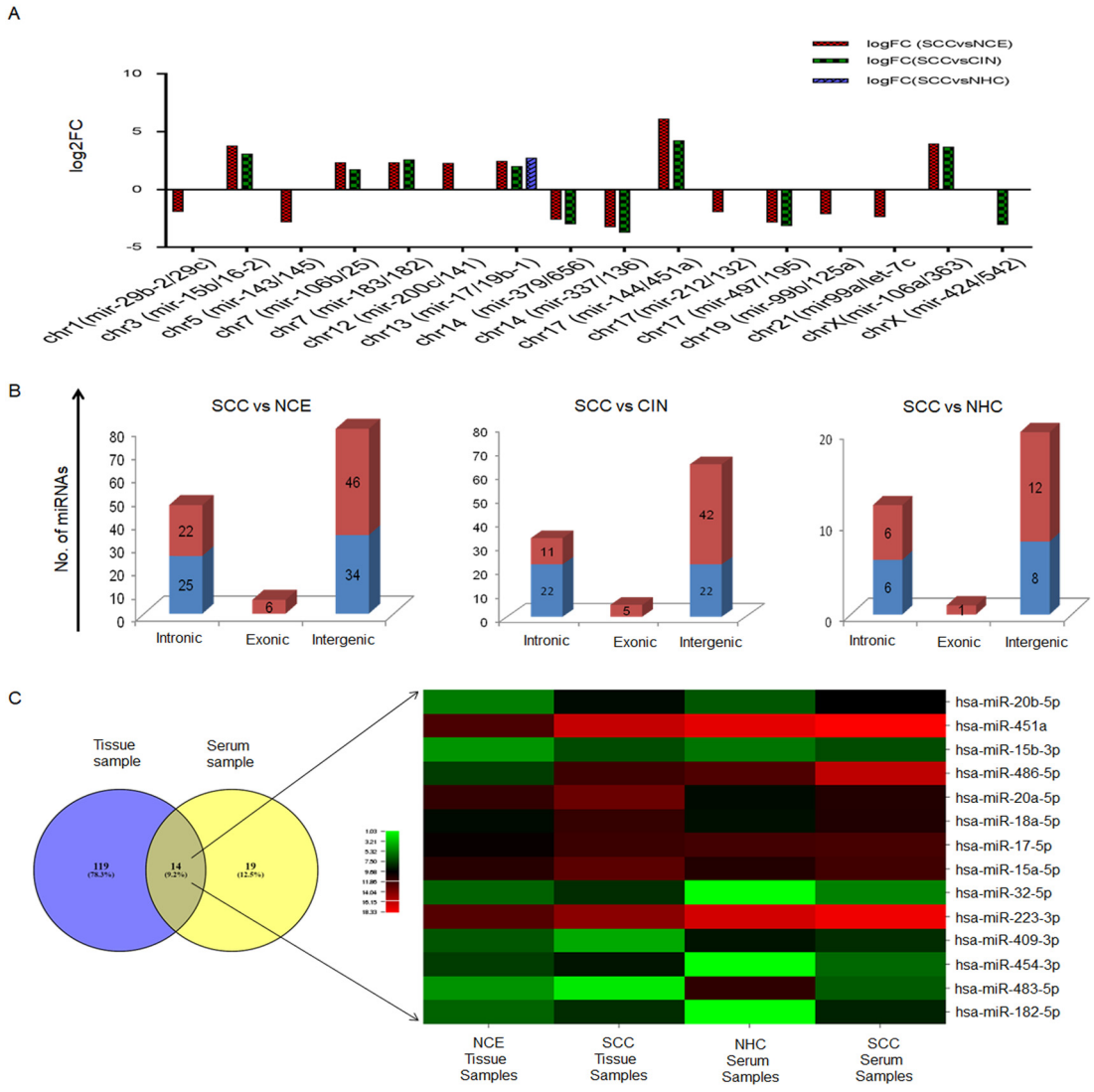

Fig. 4. A) Cluster miRNA expression. Total numbers of clusters identified in SCC and CIN compare against NCE tissue specimens and SCC against NHC serum specimens. B) The miRNA subtypes (exonic, intronic & intergenic) expression in SCC against NCE, SCC against CINI-III and SCC against NHC. Blue colour represents upregulated and red colour indicated downregulated C) Venn diagram showing the differentially expressed miRNAs (logFC>1 or <−1 with FDR < 0.05) common in tissue and serum specimens, unique to tissue and serum specimens. Heat map analysis showing the differentially expressed miRNAs common in tissue and serum specimens (logFC>1 or <−1 with FDR < 0.05). Each mature miRNA with log2-transformed expression level was used for clustering. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

specimens may unravel the role, mechanism and clinical significance in cervical cancer.

Studies have identified the utility of circulatory and tissue miRNAs profiling in cervical cancer as a biomarker. For example, Wilting et al., 2013 have performed global human miRNAs expression microarray and identified 89 altered miRNA expression in SCC ($n = 10$) compared to normal (n = 10) tissues [21]. When we compared their findings with our data, we have identified 17 common upregulated miRNAs (hsa-miR-20b, hsa-miR-17, hsa-miR-21, hsa-miR-106a, hsa-miR-106b, hsa-miR-18a, hsa-miR-363, hsa-miR-93, hsa-miR-15a, hsa-miR-185, hsa-miR-19a, hsa-miR-425, hsa-miR-25, hsa-miR-19b, hsa-miR-200c, hsa-miR-141and hsa-miR-15b) and 14 downregulated miRNAs (hsa-miR-497, hsa-miR-199b, hsa-miR-125b, hsa-miR-199a, hsa-miR-149, hsa-miR-195, hsa-miR-218, hsa-miR-376a, hsa-miR-494, hsa-miR-134, hsa-miR-99a, hsa-miR-193a, hsa-miR-100 and hsa-miR-212) in SCC compared against NCE. Also, deep sequencing of serum derived miRNAs in 133 cervical cancer and 106 age-matched control has identified novel miRNAs in cervical cancer [23]. Thus, the correlation of miRNA signatures between serum and tissue remains as a field of intense interest to understand cervical cancer progression. Toward this, we have performed genome-wide small RNAs profiling to establish miRNA expressions pattern in both serum and tissue of cervical cancer subjects and identified the differentially expressed miRNAs.

In total, we have identified 14 common miRNAs as differentially expressed in serum and tissue specimens when compared with age-matched healthy controls. Among the validated miRNAs, the hsa-miR-17-5p, hsa-miR-32-5p and hsa-miR-454-3p are upregulated and hsa-miR-409-3p ($P < 0.05$) downregulated in serum and tissue specimens. The fold difference observed in NGS for hsa-miR-17, hsa-miR-32, hsa-miR-409 and hsa-miR-454 was 2.06, 2.6, −2.4, 1.51 respectively in SCC compared against NCE samples. In qRT-PCR the fold differences were found to be 2.81, 2.3, −4.43 and 3.51 for hsa-miR-17, hsa-miR-32, hsa-miR-409 and hsa-miR-454 respectively in SCC against NCE. Although the trend in differences appear to faithfully represented between high throughput and validation experiments, it is unlikely to be precise. The difference in expression level between NGS and validation by qRT-PCR could be due to (i) use of different platforms for monitoring the miRNA expression, (ii) differences in the methods used for normalization in NGS and qRT-PCR, and (iii) biological differences due to the use of independent samples for NGS and qRT-PCR.

The hsa-miR-17-5p, a member of chr13 miRNA cluster (miR-17/92), previously reported as upregulated in cervical cancer tissue specimens,

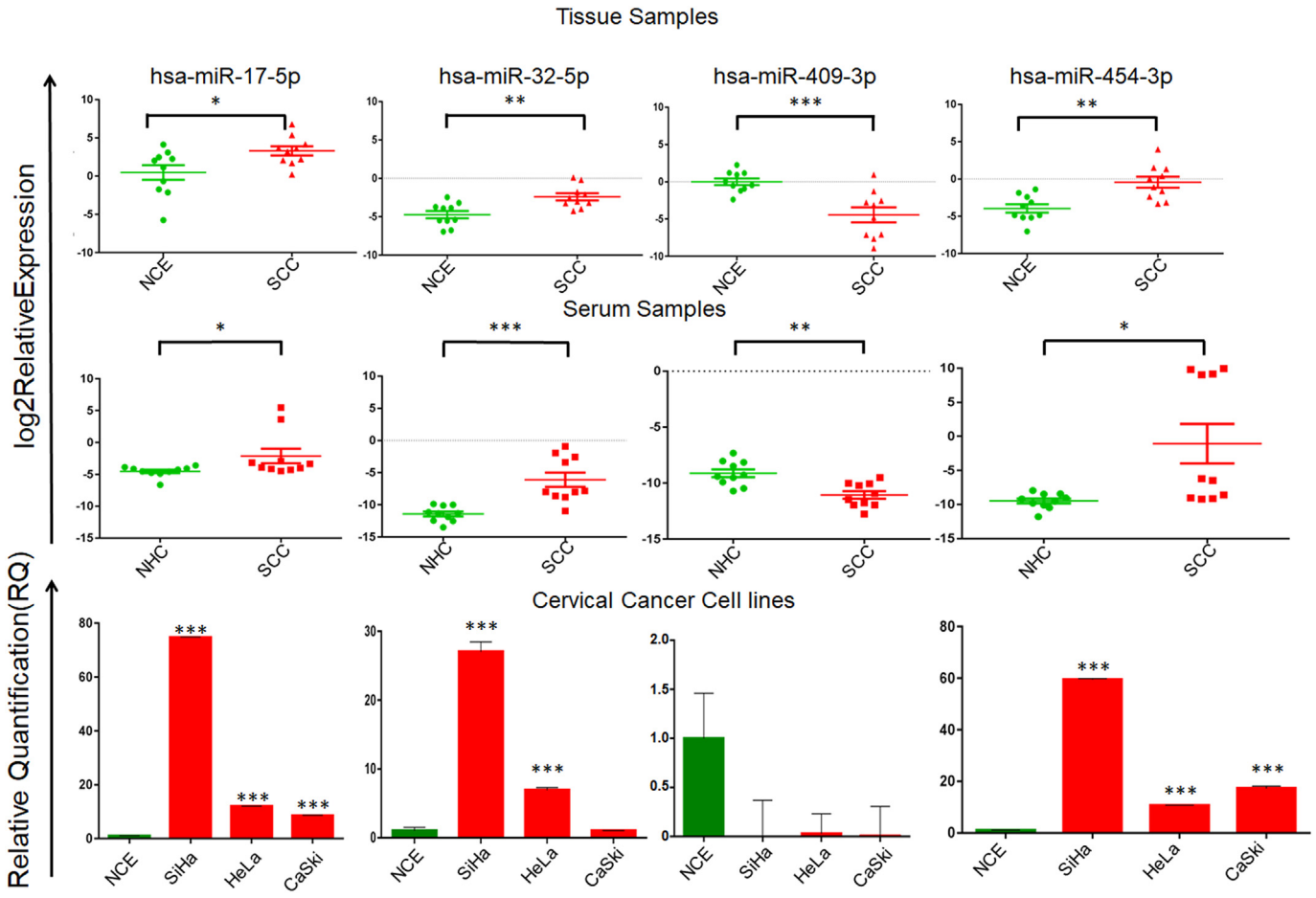

Fig. 5. The expression levels of miRNAs were determined by quantitative RT-PCR in NCE ($n = 10$) and SCC (n = 10) for tissue specimens, NHC (n = 10) and SCC (n = 10) for serum specimens and cervical cancer cell lines. Expression levels of miRNAs were normalized to RNU6B (log2 relative level) for tissue specimens and cell lines and miR-16 for serum specimens. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. The hsa-miR-454-3p showed >10 fold upregulation in SCC tissues and serum respectively. The hsa-miR-32-5p and hsa-miR-17-5p were >5 fold upregulated in the SCC tissue and serum specimens. However, hsa-miR-409-3p has shown downregulation in SCC tissues and serum specimens.

promotes tumorigenesis and metastasis by targeting E2F1 and key regulator of G1/S phase cell cycle transition [24]. As a candidate for diagnostic purpose, hsa-miR-17-5p has shown the sensitivity and specificity of 95% and 55% in the serum of cervical cancer patients [25]. The aberrant expression of hsa-miR-32-5p is associated with gastric cancer and target KLF4 transcripts [26]. Interestingly, hsa-miR-32-5p is located in 9q31.2 which is often part of cancer hotspot showing amplifications, deletions or epigenetic modifications [27]. The Kaplan-Meier survival analyses of colorectal cancer patients have shown that the higher expression of hsa-miR-32 relates to shorter survival times [28]. The hsa-miR-454-3p function as an oncogene in hepatocellular carcinoma by repressing CHD5 [29] and promotes cell proliferation in colorectal cancer by targeting *CYLD* [30]. Plasma levels of hsa-miR-454-3p are reported as a potential biomarker for glioma [31]. Patients with higher expression of hsa-miR-454-3p in hepatocellular carcinoma are associated with low overall survival [32]. The hsa-miR-409-3p, a part of chr14 miRNA cluster (miR-379/656), is silenced in most of the cancers including melanoma [33] and promotes metastasis in colorectal cancer [34]. Interestingly, all the miRNAs validated here showed similar expression pattern in both cervical serum and tissue specimens suggesting its potential as a minimally invasive biomarker for cervical cancer. The hsa-miR-32-5p and hsa-miR-409-3p showed maximum sensitivity and specificity of >80% in both tissue and serum specimens (Fig. S1).

Target prediction analyses showed *ST18* (Suppressor of Tumorigenicity 18) one of the putative binding sites for hsa-miR-454-3p. In breast cancer, it was found to be significantly downregulated and ectopic expression of *ST18* in breast cancer cells inhibits colony formation in soft agar and xenograft tumor formation [35]. It has also been shown that *ST18* regulates pro-apoptotic and pro-inflammatory gene expression in fibroblast and has pro-apoptotic effect on pancreatic B-cells [36]. These data suggested that *ST18* behaves as suppressor of tumor growth. However, its role in cervical cancer is yet to be established. Our study shows that *ST18* is downregulated in SiHa cells due to upregulation of hsa-miR-454. Further, *MTF2* (Metal Regulatory Transcription Factor) is reported to be regulated by hsa-miR-409-3p *via* binding to its 3′UTR region. *MTF2* is a member of polycomb-like (PCL) proteins which are substoichiometric components of the Polycomb-repressive complex2 (PRC2) and plays a vital role in cancer [37]. Ectopic expression of PCL1–3 increases the recruitment of the PRC2 complex and H3K27me3 deposition on the INK4A tumor suppressor gene locus [37]. The expression of *MTF2* was shown to be upregulated in colorectal cancer hence may promote oncogenicity in cancer [38]. Additionally, forced expression of hsa-miR-409 mimics and hsa-miR-454 inhibitor mimics have decreased the expression of *MTF2* and *ST18* respectively and inhibited the cell proliferation.

Several miRNAs reported here are present as clusters and have similar pattern of expression. These include 3 miRNAs from chr13 cluster (miR-17/92) which are upregulated in SCC serum and tissue specimens and are consistent with findings with other cancers as well [39]. Therefore, understanding of the coordinated regulation, selective activation or inactivation and the impact of these clusters on the different targets is important to delineate the biological variations and to decipher the clinical implications. Interestingly, in our study, miRNAs from exonic region were all downregulated in cervical cancer tissue and serum

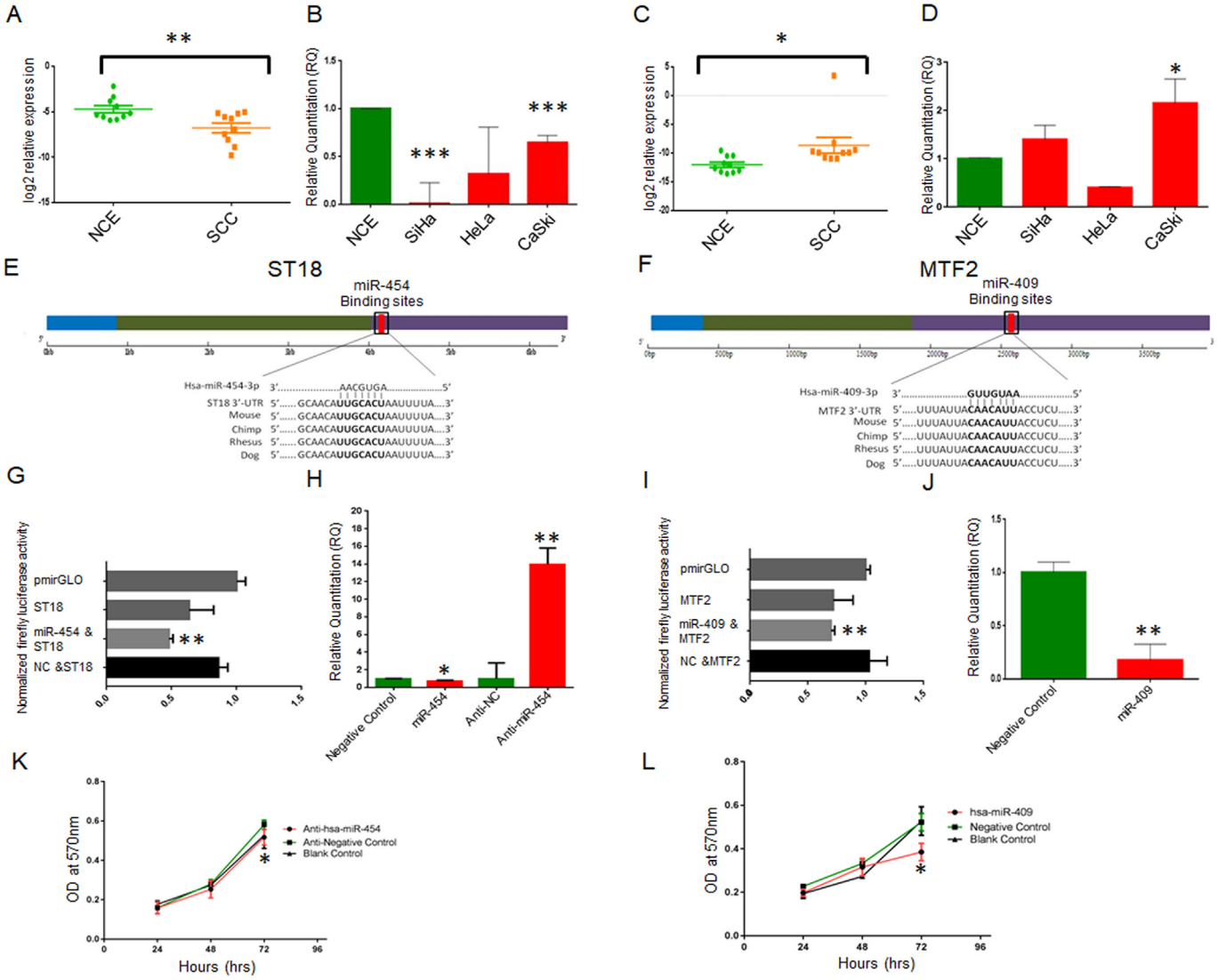

Fig. 6. Expression analysis of *ST18* (A and B) and *MTF2* (C and D) in clinical specimens ($n = 20$) and cervical cancer cell lines. (E and F) hsa-miR-454-3p and hsa-miR-409-3p binding sites on *ST18* and *MTF2* in 3′-UTR and their conservational analysis. (G and I) Dual luciferase assay indicated that hsa-miR-454-3p and hsa-miR-409-3p downregulated the expression of *ST18* and *MTF2 via* binding to its 3′-UTR respectively. (H and J) In comparison to negative control, hsa-miR-454-3p and hsa-miR-409-3p inhibited the mRNA levels of *ST18* and *MTF2* respectively. (K and L) Transfection with anti-miR-454-3p and hsa-miR-409 mimics significantly decreased the proliferation of SiHa cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

specimens. Further studies on the regulation of biogenesis of intergenic and exonic miRNAs will give better understanding of molecular mechanism underlying progression of cervical cancer. The small RNA sequencing data has revealed variability in miRNA biogenesis with canonical mature miRNA sequences which comprise multiple sequence isoforms [40]. Among all, 3′-modifications were predominant in both tissue and serum specimens followed by 5′-trimming. Taken together, these isomiRs might alter the target recognition which requires further investigation.

In summary, our study has identified miRNA expression in tissue and serum of both normal and tumor specimens. Further, we have identified differentially expressed miRNAs of tissue which can be even be identified from serum specimens. The miRNAs identified by us showed good sensitivity and specificity to distinguish normal from SCC specimens and has the potential to be used as a minimally invasive marker. This comprehensive investigation of miRNAs in cervical cancer has generated baseline data which can be used for various translational researches. Further, studies including matched tissue and serum needs to be undertaken in large cohorts of samples to evaluate robust miRNA signature for minimally invasive diagnostic and therapeutic purposes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2019.08.012.

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Declaration of competing interest

All authors report no conflicts of interest.

Author contributions

Vaibhav Shukla and Vinay Koshy Varghese processed the small RNA sequencing. Vaibhav Shukla, Vinay Koshy Varghese, Shama Prasada

Kabekkodu, Sandeep Mallya, Pradyumna Jayaram and Sanjiban Chakrabarty analyzed and interpreted the data. Vaibhav Shukla, Vinay Koshy Varghese, Shama Prasada Kabekkodu and Kapaettu Satyamoorthy discussed the manuscript. Deeksha Pandey, Krishna Sharan, and Sourjya Banerjee monitored the patients who participated in this project. Kapaettu Satyamoorthy designed and supervised the study.

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