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DIFFERENTIAL miRNA EXPRESSION PROFILING IN HPV-16 POSITIVE CERVICAL CANCER

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ABSTRACT: Cervical cancer (CC) is a leading cause of cancer in developing countries like India. Human Papillomavirus (HPV) is one of the major risk factor for CC. Increasing evidence suggests that HPV infection dysregulates miRNA expression. However, a comprehensive molecular HPV-miRNA network is still unknown. HPV 16 positive type plays an important role in CC progression. In this present study, we desired to delineate the potential HPV-miRNA regulatory pathway contributing to the pathogenesis of HPV 16 mediated CC. In a previously published data, we found that miR-375 is one of the most downregulated miRNA during HPV infection and -33.33 fold decreased in cervical squamous cell carcinoma when compared to control. We further confirmed the miR-375 expression in cervical cancer by an integrated meta-analysis. Microarray GSE19611, GSE32724 and GSE81137 datasets were retrieved from the Gene Expression Omnibus. miR-375 significantly downregulated in the squamous cervical patient when compared to the normal cervix. In C33a cervical cancer cells, miR-375 expression was upregulated when compared to SiHa, CaSki and HeLa. Furthermore, we analysed HPV-16 mediated miRNA expression in CC patients and healthy population, top 10 upregulated and downregulated miRNAs were identified respectively. miR-375 was identified as one of the dysregulated miRNA by HPV-16 and target a novel oncogene AEG-1. Ectopic expression of miR-375 and silencing of its target AEG-1 induced apoptosis through nuclear fragmentation, mitochondrial membrane depolarization and ROS generation in CC cell lines. Our integrative analysis shows that miR-375 is a potential target for HPV-16, which serves distinct role in CC progression.

KEYWORDS: Human papillomavirus 16; miR-375; AEG-1; Gene Ontology; Microarray; Gene Expression Omnibus.

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1.INTRODUCTION

Cervical cancer (CC) is one of the commonest cancer to women in the world especially in countries like India which have a high incidence of Human Papillomavirus (HPV) infection. Apart from HPV, it may be caused by other factors that lead to CC of the cervix such as lifestyle, and smoke [1]. Cancer that develops in the ectocervix is called squamous cell carcinoma, and around 80-90% of cervical cancer cases (more than 90% in India) are of this type [2]. HPV is the main risk factor for the development of cervical cancer, HPV DNA has been found in almost all cases of invasive cervical cancer [3]. There are more than 100 types of HPV have been identified and these viruses can be classified into high-risk or low-risk groups according to their oncogenic potential. High-Risk HPV (HR-HPV) types 16 and 18 strains are associated with more than 90 % of CC [4-5]. MicroRNAs (miRNAs), a class of small regulatory noncoding RNAs of 21-25 nucleotides in length that negatively regulate target gene expression in cellular process such as cellular proliferation, differentiation, and apoptosis through translational repression or mRNA degradation, which depends on their complementation with the 3' UTR (Untranslated Region) of target mRNA [6-8]. miRNA expression has been deregulated in a different types of human diseases including cancer. However, it remains unclear whether altered miRNA expression is a cause or consequence of many pathological processes [9]. Epigenetic alterations are also a crucial process of miRNA dysregulation in cancer. However, whether this mechanism contributes to the miRNA downregulation in CC has been largely unknown. Recent evidence suggests that HPV deregulates tumor suppressor genes and induced cancers [10-11]. With the advent of microarray experiments, few studies anticipate the differential expression of miRNA in HPV mediated CC, but failed to focus on the deregulated signaling pathways. In this present study, the miRNA expression profile from CC patients, CC cell lines and HPV 16 positive CC patients were downloaded from Gene Expression Omnibus (GEO, GSE19611, GSE32724 and GSE81137). Then, we successively analysed differential miRNA expression, Gene Ontology (GO) functional annotation, target gene prediction, signaling pathways. Further, the miR-375 functions in apoptosis, mitochondrial membrane potential and ROS generation were validated using Ehidium Bromide, JC-1 and DCF-DHA staining. Our results demonstrated that miR-375 is an ideal target for HPV-16 and plays an important role in CC progression.

2. MATERIALS AND METHODS

2.1. miRNA microarray data analysis

miRNA expression datasets for normal and HPV positive cervical cancer were taken for further

analysis. GSE19611, GSE32724 and GSE81137 datasets were downloaded from Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/), a functional genomics data repository. The normalized data downloaded for each platform were annotated with official miRNA symbol using appropriate platform annotation files. GSE19611 data sets were used to find out miR-375 expression in cancer patients and GSE32724 data sets were used to find out miR-375 expression in cervical cancer cell lines. Top 10 up and down regulated genes were chosen from GSE81137 data sets with a cutoff criteria fold change $\geq$ 1.5. Meta-analysis was performed to identify potential miRNA among the normal and HPV 16 positive cervical cancer patients with the stringent criteria.

2.2. miR-375 target prediction

The potential dysregulated miR-375 target genes were predicted by miRNet (http://www.mirne t.ca/), which is an easy-to-use comprehensive tool integrated data analysis and used to predict miRNA-375 target gene AEG-1 as well as its target regions and AEG-1 regulated genetic network generated with GeneMANIA online tool.

2.3. Functional annotation and pathway enrichment analysis

Panther online program, the database for annotation, visualization and integrated discovery (Panther, http://www.pantherdb.org/), is an extensive tool for understanding biological meaning behind multiple genes [12]. In our study, Panther Gene Ontology was used to perform Gene Ontology (GO) and functional annotation is a capability of hierarchal representation of gene in three divisions includes cellular component, Molecular function and biological process to predict potential dysregulated miRNAs target genes.

2.4. Cell Culture

Cervical cancer cell lines HeLa, SiHa, CaSki, and C33A were purchased from the National Center for Cell Sciences, Pune, India and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin antibiotics (Invitrogen, Carlsbad, CA, USA). All the cells were cultured in a humidified incubator with 5% CO_2 at 37^0C.

2.5. Transfection of miRNA mimic, inhibitor, siRNA and their corresponding negative controls

Cervical cancer cells were seeded into six-well plate (1 X 10^6) after reached 60% confluence. For siRNA transfection, AEG-1 siRNA and their negative control siRNA were purchased from Sigma Aldrich, USA and for miRNA transfection, miR-375 mimic, miR-375 inhibitor and their related negative control (Applied Biosystems, CA, USA) were transiently transfected into cells at a concentration of 20 nM using Lipofectamine RNAiMAX Reagent (Invitrogen). The mock control cells were treated with transfection reagent alone and the cells were maintained for 48 h after transfection.

2.6. Acridine orange/ethidium bromide (AO/EB) dual staining

Cells were grown in a 6 well plate and transfected with miR-375 mimic, miR-375 inhibitor, AEG-1 siRNA and their corresponding controls for 48 h. Then medium was removed and cells were washed

with PBS and stained with 10 µl AO/EB (1 mg/ml) for 10 min. Morphological changes were visualized with a FLoid cell imaging station (Life Technologies, USA).

2.7. DAPI staining

Chromatin condensation and nuclear fragmentation were analyzed using DAPI staining. Cells were cultured in a 6 well plate and transfected with miR-375 mimic, miR-375 inhibitor, AEG-1 siRNA and their corresponding controls for 48 h. Then medium was removed and cells were washed with PBS and stained with 15 µl DAPI (100 µg/ml) and incubated for 30 min at 37^0C. The stained cells were visualized with a FLoid cell imaging station (20X) (Life Technologies, USA).

2.8. Determination of mitochondrial membrane potential (Δψm)

The alterations of mitochondrial membrane potential in CC cells were analyzed using a JC-1 fluorescent probe. Briefly, CC cells were transfected with miR-375 mimic, miR-375 inhibitor, AEG-1 siRNA and their corresponding controls for 48 h. CC cells were then washed with PBS and stained with JC-1 for 1 h in dark at 37^0C. Stained cells were visualized with a FLoid cell imaging station (20X) (Life Technologies, USA).

2.9. Detection of intracellular ROS accumulation

DCFH-DA stain was used to detect the intracellular reactive oxygen species accumulation. DCFH-DA readily diffuses into cells and produce DCFH which is further oxidized by intracellular ROS to transform non-fluorescent DCFH to highly fluorescent DCF. Briefly, CC cells were plated onto 6 well plates and transfected with miR-375 mimic, miR-375 inhibitor, AEG-1 siRNA and their corresponding controls for 48 h. After incubation, the medium was removed and cells were washed with PBS and stained with 100 µl DCFH-DA (50 µM) for 30 min in dark at 37^0C. The fluorescence was detected by a FLoid cell imaging station (20X) (Life Technologies, USA).

3. RESULTS AND DISCUSSION

3.1. miR-375 differentially expressed in cervical cancer

In a previous research [13], 21 miRNAs were dysregulated in cervical squamous carcinoma cell (CSCC) and head and neck squamous carcinoma cell (HNSCC). Among these 21 differential expressed miRNA, we found that miR-375 was decreased in -33.33 fold changes in cervical squamous cell carcinoma when compared to control [13]. We retrieved the dataset from GEO and analysed the miR-375 expression in Normal, Squamous cervical cancer (SCC), Low-grade squamous intraepithelial lesion (LSIL) and High-grade squamous intraepithelial lesion (HSIL) by using GEO2R. (Fig. 1) illustrates that miR-375 expression was decreased in LSIL, HSIL and SCC when compared to the normal cervix. Furthermore, miR-375 expression was decreased in SCC when compared to LSIL and HSIL. However, there were no changes miR-375 expression in both LSIL and HSIL [14]. From this result, it was observed that miR-375 drastically downregulated in SCC.

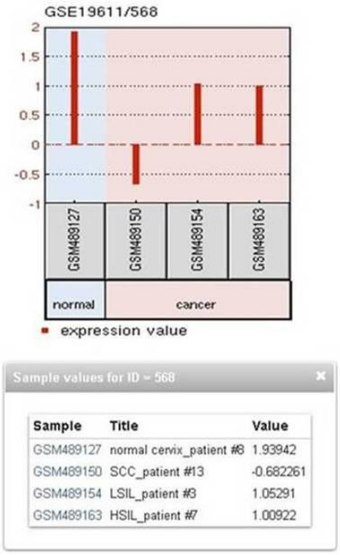


Figure 1. miR-375 expression in Normal cervix and SCC, LSIL, HSIL. B).miR-375 expression in HPV positive and HPV negative cervical cancer cell lines

3.2. miR-375 differentially expressed in cervical cancer cell lines

In a previous study [15], they were used 4 different types of human cervical cancer cell lines (C33A-HPV-ve, HeLa-HPV 18+ve, SiHa-HPV 16+ve and CaSki-HPV 16+ve) and all the cells were divided into two groups and one group was normal control, which means without treatment and another one group was treated with 10 Amol/L 5-aza-2'-deoxycytidine. miRNA expression analysis raw data were submitted to Gene Expression Omnibus (GEO) with the accession number of GSE32724 [15]. We retrieved only the untreated 4 cervical cancer cell lines dataset from GEO and analysed the miR-375 expression and analysed the miR-375 expression by using GEO2R. Interestingly, miR-375 drastically downregulated in HPV- positive cell lines such as HeLa, SiHa and CaSki (Fig. 2) when compared to HPV - negative cell line (C33A).

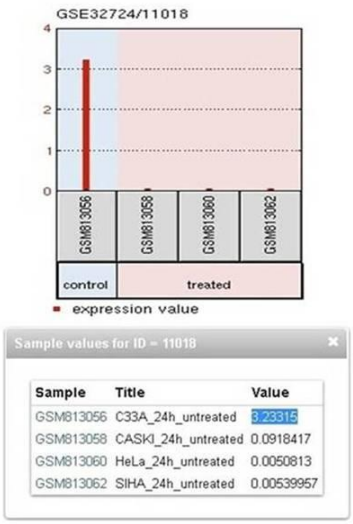


Figure 2. miR-375 expression in HPV positive and HPV negative cervical cancer cell lines

3.3. miR-375 differentially expressed in healthy and HPV positive patients

In a previous results, miR-375 significantly downregulated in cervical cancer patient [14] as well as in cervical cancer cell lines [15]. Moreover, how miR-375 dysregulated in cervical cancer is still unknown, HPV infection is a major concern for developing cervical cancer. miRNA expression profile in healthy and HPV/HIV associated cervical cancer was already submitted by vijayamma et al, (2016) in Gene Omnibus (Accession number is GSE81137). So, we aimed to differentiate the miR-375 expression in normal and HPV positive cervical cancer patients. We further retrieved two datasets which contain a healthy population and HPV 16 positive population only and then analysed the top 10 upregulated (Table. 1A) and downregulated (Table. 1B). miRNAs Interestingly, miR-375 expression was -1.8 fold decreased in HPV 16 patients when compared to healthy population.

Table 1. A). Top 10 upregulated miRNA in healthy and HPV 16 positive cervical cancer population**. B).** Top 10 down regulated miRNA in healthy and HPV 16 positive cervical cancer population.

A). Up regulated miRNA

S. No.	miRNA	LogFC	p. Value
1	hsa-miR-152_st	1.982865	0.000943
2	hsa-miR-10b_st	1. 507633	0.059407
3	hsa-miR-205-star_st	1.368035	0.02808
4	hsa-miR-486-5p_st	1.321997	0.091852
5	hsa-miR-196a_st	1.317194	0.010543
6	hsa-miR-151-3p_st	1.285343	0.027521
7	hsa-miR-151-5p_st	1.273381	0.051262
8	hsa-miR-502-3p_st	1.23336	0.053486
9	hsa-miR-135b_st	1.222185	0.009064
10	hsa-miR-151b_st	1.159291	0.040424

B). Down regulated miRNA

S. No.	miRNA	LogFC	p. Value
1	hsa-miR-3647-5p_st	-2.8059	0.0000196
2	hsa-miR-3148_st	-2.27526	0.0086399
3	hsa-miR-375_st	-1.80028	0.0185566
4	hsa-miR-3153_st	-1.70286	0.0101313
5	hsa-miR-5096_st	-1.7028	0.0075486
6	hsa-miR-223_st	-1.70069	0.0337928
7	hsa-miR-27a-star_st	-1.63141	0.0629198
8	hsa-miR-491-3p_st	-1. 5674	0.0129904
9	hsa-miR-223-star_st	-1. 55601	0.0606023

10	hsa-miR-593-star_st	-1. 54845	0.001478

3.4. AEG-1 genetic network

Genetic interaction network of AEG-1 was integrated and generated with the help of the GeneMANIA online tool (Fig. 3), which predicts that AEG-1 is interacted with various genes which were involved in apoptosis, cell cycle, angiogenesis, autophagy, chemoresistance and PI3K/AKT signaling pathway.

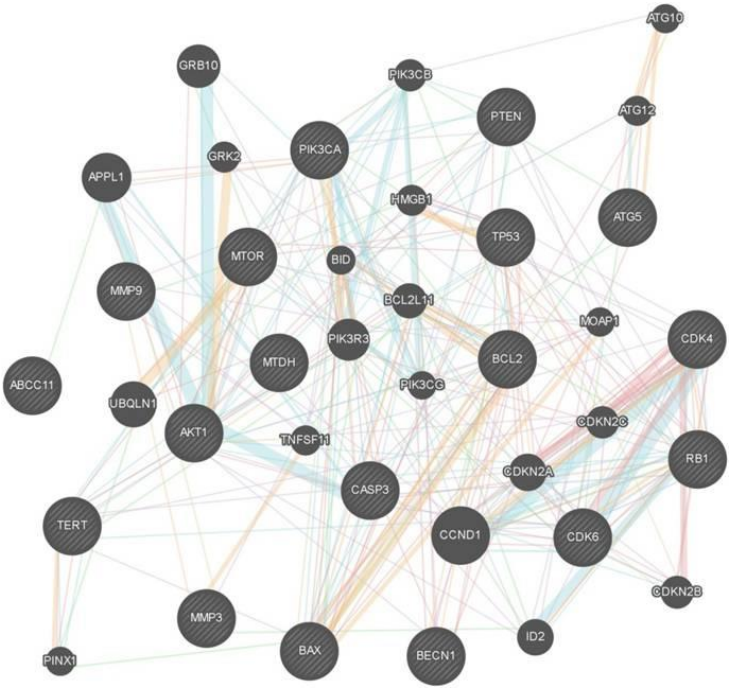
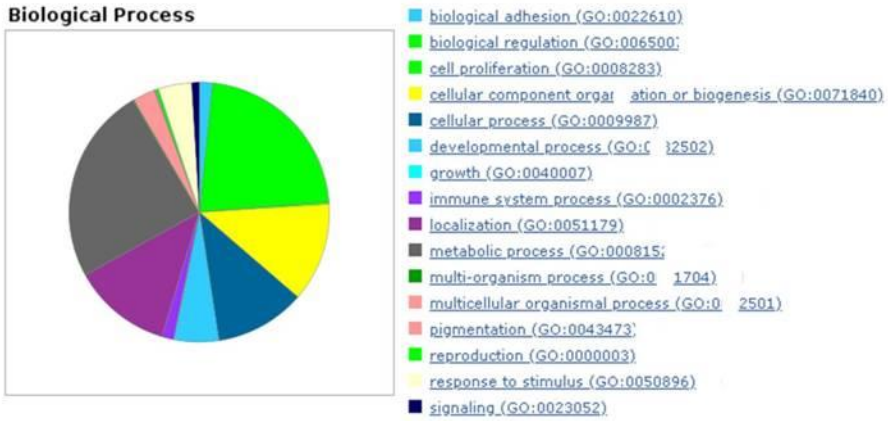


Figure 3. Genetic network of AEG-1 (MTDH) and associated genes generated with the GeneMANIA online tool.

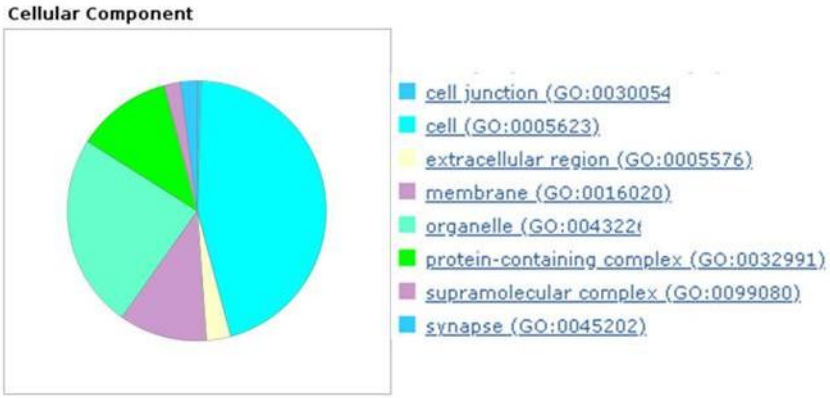
3.5. Identification of signaling pathway

miR-375 targets 481 different genes and regulates the biological process, molecular function and cellular component. miR-375 targeted genes involved in various biological signaling pathways, particularly in cancer: Angiogenesis pathway, Apoptosis pathway, Cadherin signaling pathway, EGF receptor signaling pathway, JAK/STAT signaling pathway, Notch signaling pathway, p38 MAPK pathway, p53 pathway, PI3 kinase pathway, Ras pathway, TGF-beta signaling pathway, Ubiquitin proteasome pathway, VEGF signaling pathway and Wnt signaling pathways (Fig. 4 A-E).

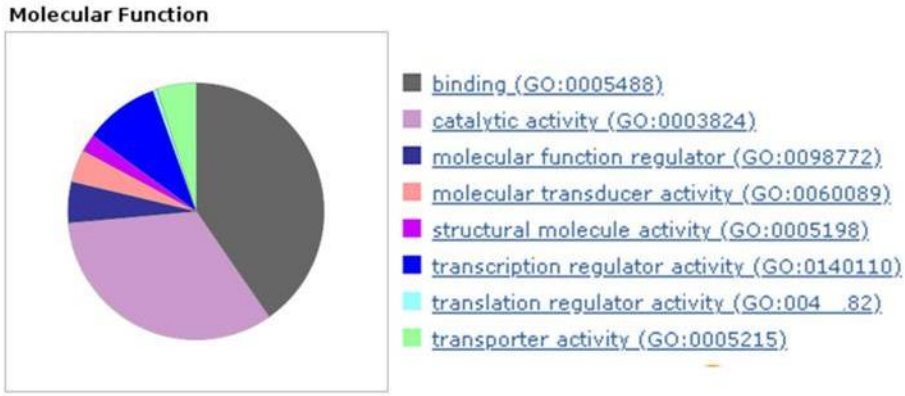
A)



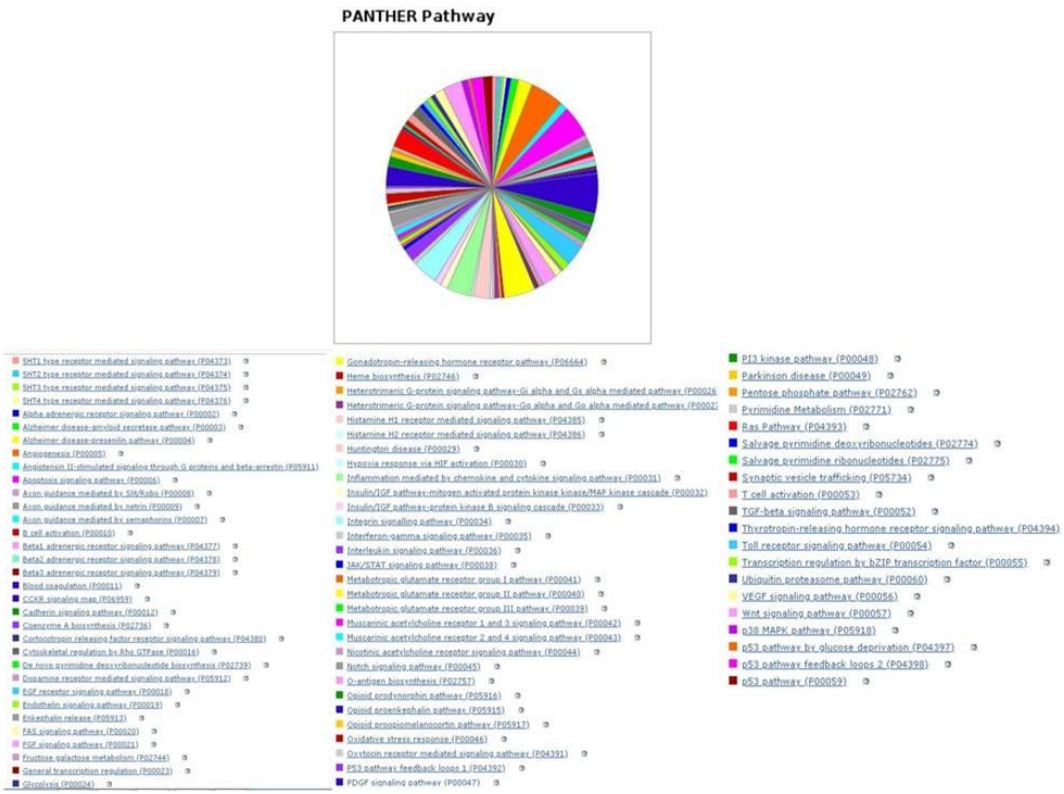
B)



C)



D)



E)

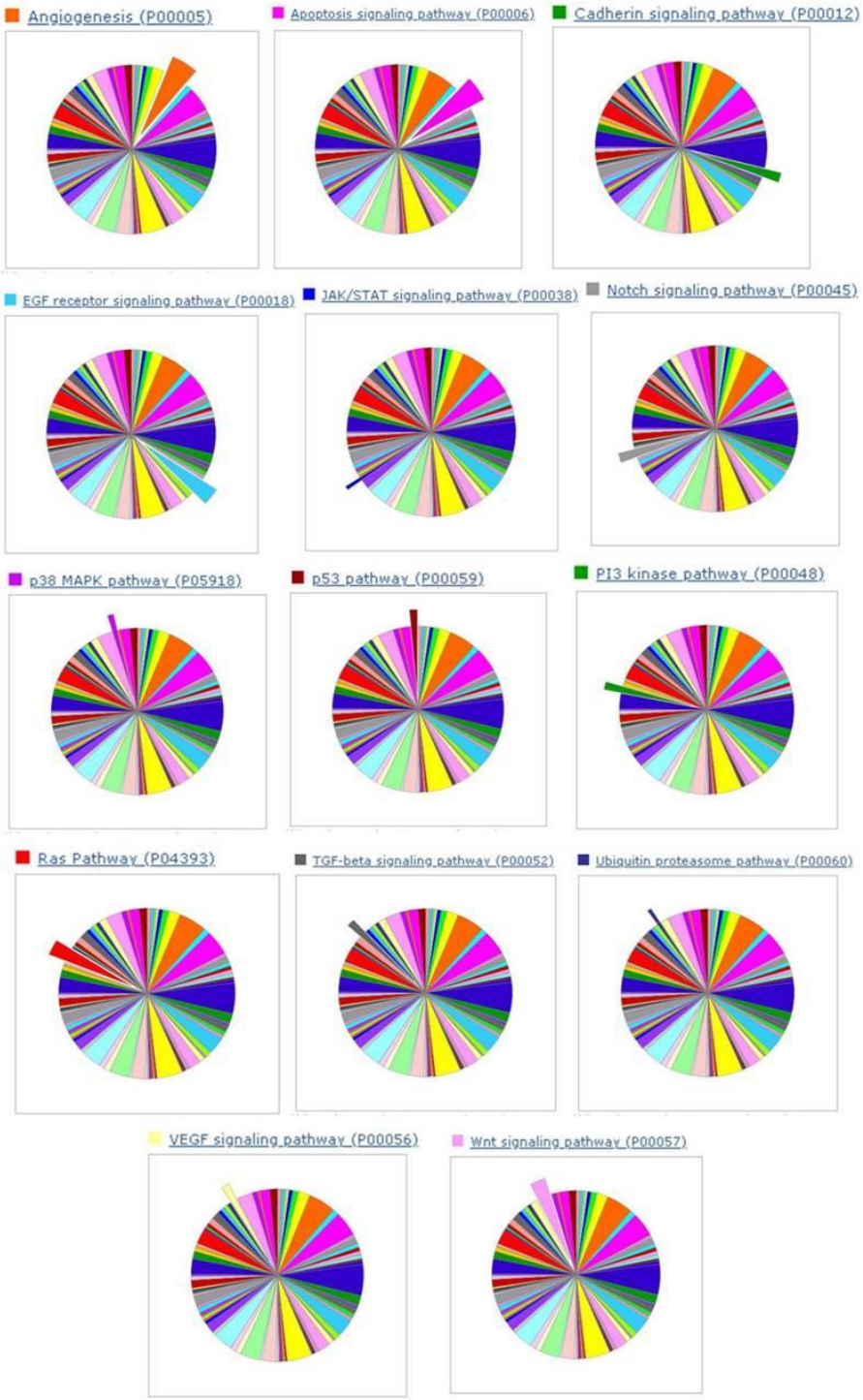


Figure 4. The GO annotation for the miR-375 target genes.**A)**. Biological process **B)**. Cellular component **C)**. Molecular Functions **D)**. Biological pathways. miR-375 targets481 genes regulatedbiological pathways. **E)**. Cancer specific signaling pathway analysis.

3.6. AEG-1 is a direct target of miR-375

We utilized TargetScan and miRNet database to predict the targets of miR-375 and its binding sites to analyse the molecular mechanisms by which miR-375 regulates CC cellular proliferation, invasion and migration. We observed that miR-375 binds at AEG-1 3'UTR region (base pair of 1440 to 1461) (Fig. 5). Moreover, we found top 10 dysregulated miRNA in healthy and HPV 16 positive

patients (Table. 2). Further, we identified which miRNAs targeted AEG-1 by using TargetScan prediction tool. Surprisingly, miR-375 having 98 context score when compared to other miRNAs. From this result, miR-375 significantly downregulated in HPV mediated cervical cancer and it directly targets AEG-1.

Figure 5: miR-375 target region of AEG-1

Table 2.miRNAs target AEG-1

S. No	miRNA	Position in the UTR	Seed match	Context ++ score	Context ++ score percentile	Weighted context ++ score	Conserved branch length	Pct
1	hsa-miR-3647-5p	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2	hsa-miR-3418	817-824	8mer	-0.03	60	0.03	0	N/A
3	hsa-miR-375	1454-1461	8mer	-0.21	98	-0.21	0	<0.1
4	hsa-miR-3153	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5	hsa-miR-5096	2221-2227	7mer-m8	-0.02	6 N/A	0	0	N/A
6	hsa-miR-223	N/A	N/A	N/A	N/A	N/A	N/A	N/A
7	hsa-miR-27a	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8	hsa-miR-491-3p	451-457	7mer-m8	-0.13	71	-0.13	0.374	N/A
9	hsa-miR-223*	451-457	7mer-m8	-0.13	71	-0.13	0.374	N/A
10	hsa-miR-593-3p	5010-5016	7mer-m8	-0.01	24	0	0.01	N/A

3.7. miR-375 induces apoptosis and morphological changes in CC cell lines

CC cells were transfected with miR-375 mimic, miR-375 inhibitor, AEG-1 siRNA and their corresponding controls. After 48 h of post-transfection, cells were stained with AO/EB. Live cells nuclei intact and appeared in green colour. Dead cells appeared in bright orange colour. There were no apoptotic cells and morphological changes observed in corresponding controls which appeared in green colour (Fig. 6)

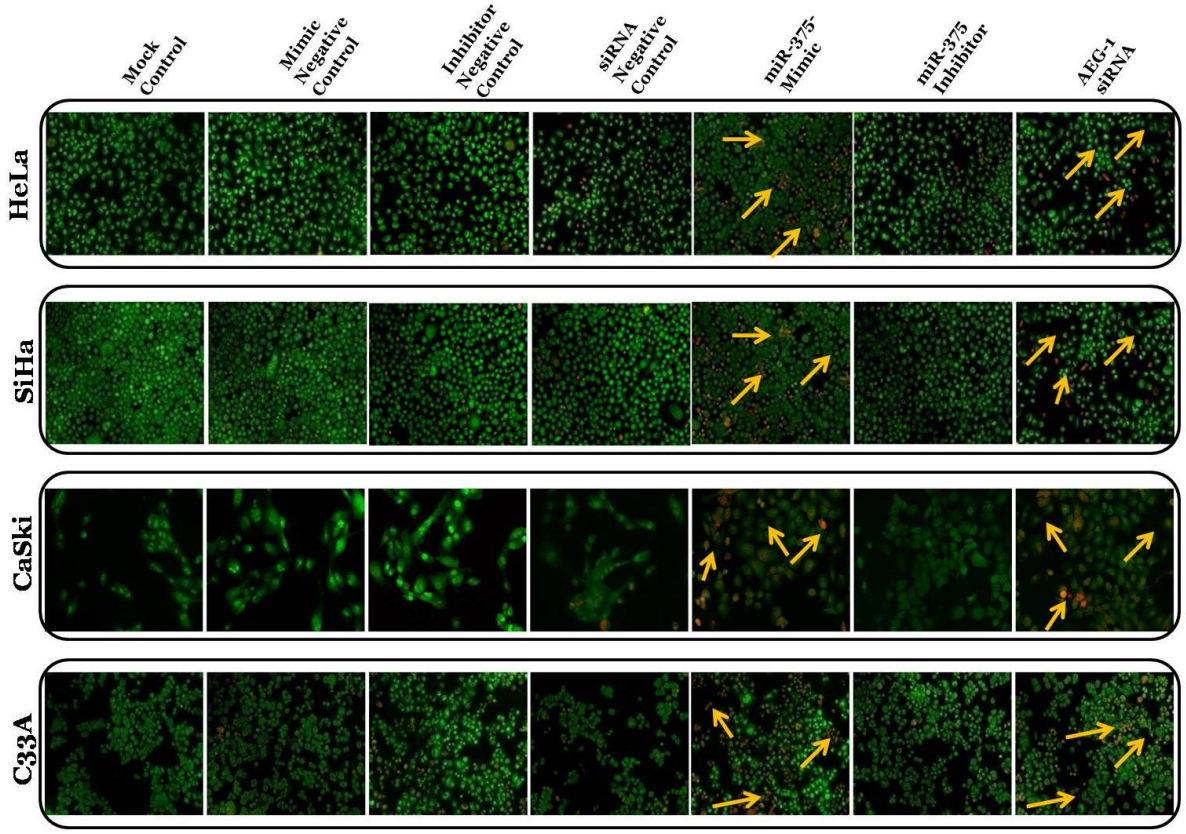


Figure 6. Apoptosis and Morphological changes in CC cells. Apoptotic cells and Morphological changes in CC cells (indicated by arrows) were visualized under FLoid cell imaging station (20X magnification)

3.8. miR-375 induces DNA damage and nuclear condensation in CC cells

CC cells were transfected with miR-375 mimic, miR-375 inhibitor, AEG-1 siRNA and their corresponding controls for 48 h. DNA damage was analyzed by using DAPI (DNA-binding stain). Mock control, miR-mimic negative control, miR-inhibitor negative control and siRNA negative control transfected cells appeared blue and had uniform nuclear shape there were no nuclear condensation and DNA damage. However, most of the miR-375 mimic and AEG-1 siRNA transfected CC cells showed damaged DNA and fragmented nuclei (Fig. 7). In contrast, miR-375 inhibitor transfected cell does not show any DNA damage. These results confirmed that miR-375 directly damaged the DNA and induced apoptosis in CC cells.

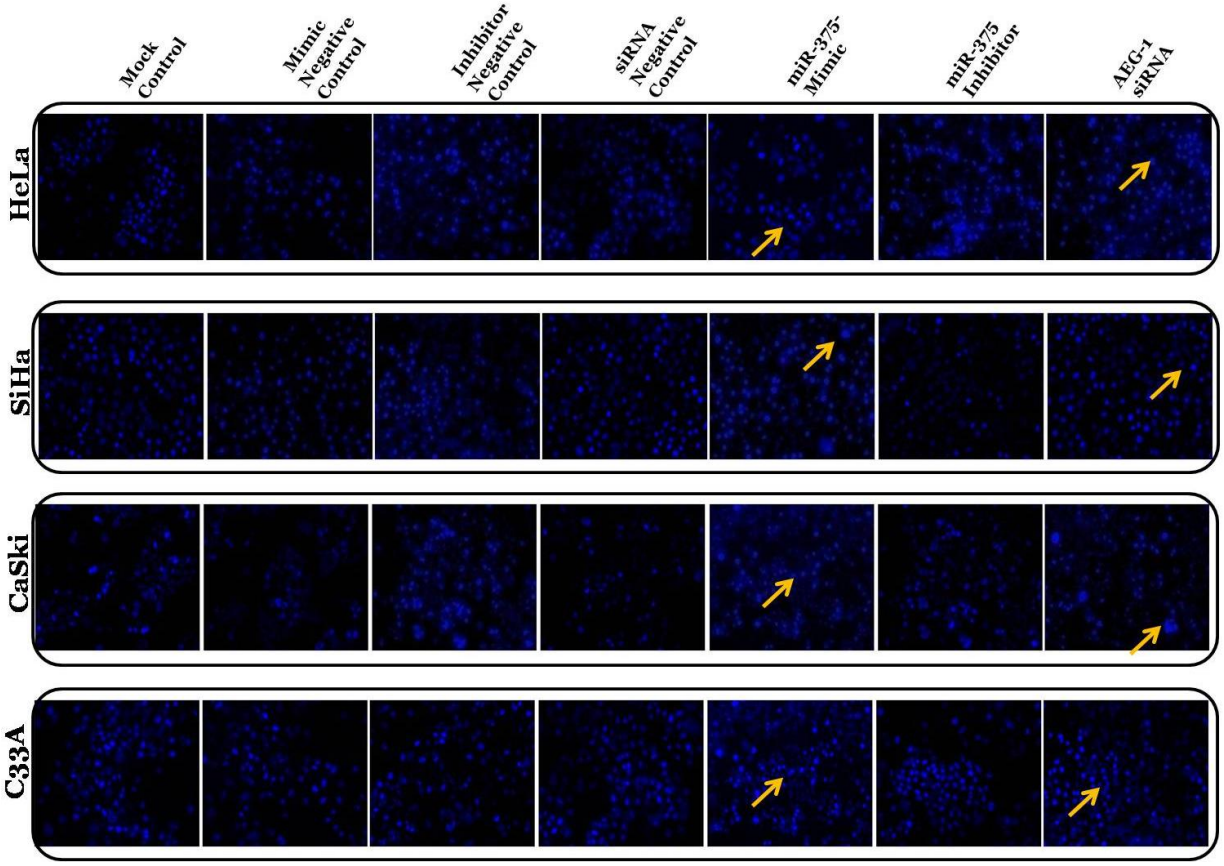


Figure 7. Nuclear fragmentation and chromatin condensation in CC cells. Transfected cells were stained with DAPI and nuclear fragmentation was observed with a Floid cell imaging station (20 X). Control cells showed healthy nuclease and miR-375 mimic, AEG-1 siRNA treated cells showed nuclear fragmentation and chromatin condensation.

3.9. miR-375 induces loss of mitochondrial membrane potential (Δψm)

Loss of mitochondrial membrane potential is indicated early apoptosis in cancer cells. CC was transfected with miR-375 mimic, miR-375 inhibitor, AEG-1 siRNA and their corresponding controls for 48 h and stained with JC-1. InmiR-375 mimic and AEG-1 siRNA transfected cells, the Δψm was lost and JC-1 aggregated; so, cells appeared green colour nuclei (Fig.8).

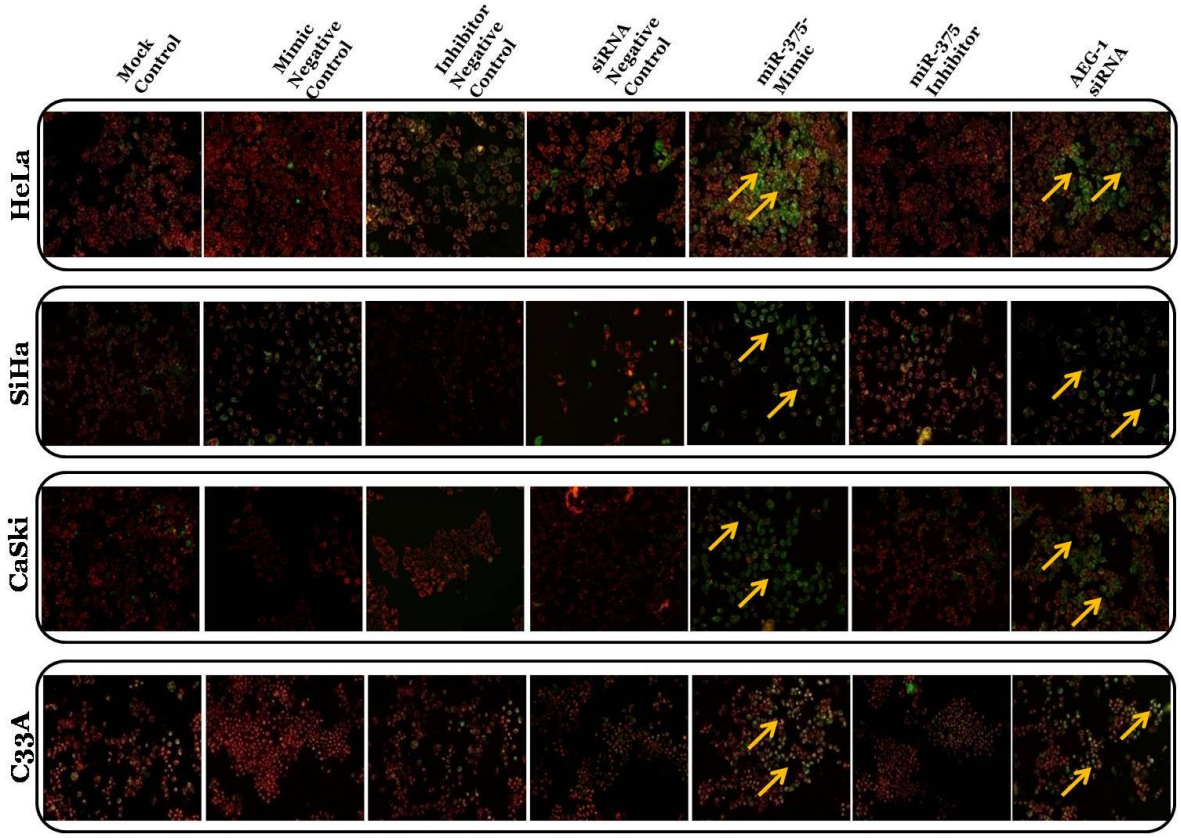


Figure 8. Determination of mitochondrial membrane potential (Δψm).CC cells were stained with JC-1 and visualized under a Floid cell imaging station (20X). Depolarization of mitochondrial membrane was indicated by the conversion of red to green (indicated by arrows). which means mitochondrial membrane was depolarized. There were no changes in Δψm in miR-375 inhibitor and control RNAi oligonucleotides transfected cells which had a red fluorescence which means mitochondrial membrane was energized. These results indicated that miR-375 induced the loss of Δψm and activated the mitochondrial-mediated apoptotic pathway in CC cells.

3.10. miR-375 induces ROS generation in CC cells.

Determination of the intracellular ROS generation in cancer cells by using ROS indicator DCFH-DA stain. The ROS level was analyzed in miR-375 mimic, miR-375 inhibitor, AEG-1siRNA and their corresponding controls transfected CC cells for 48 h by DCFH-DA. miR-375 mimic and AEG-1 siRNA transfected cells produced intracellular ROS and appeared green and the ROS levels in miR-375 inhibitor and control RNAi oligonucleotides transfected cells were also examined. miR-375 increased ROS generation when compared to the control cells (Fig. 9).

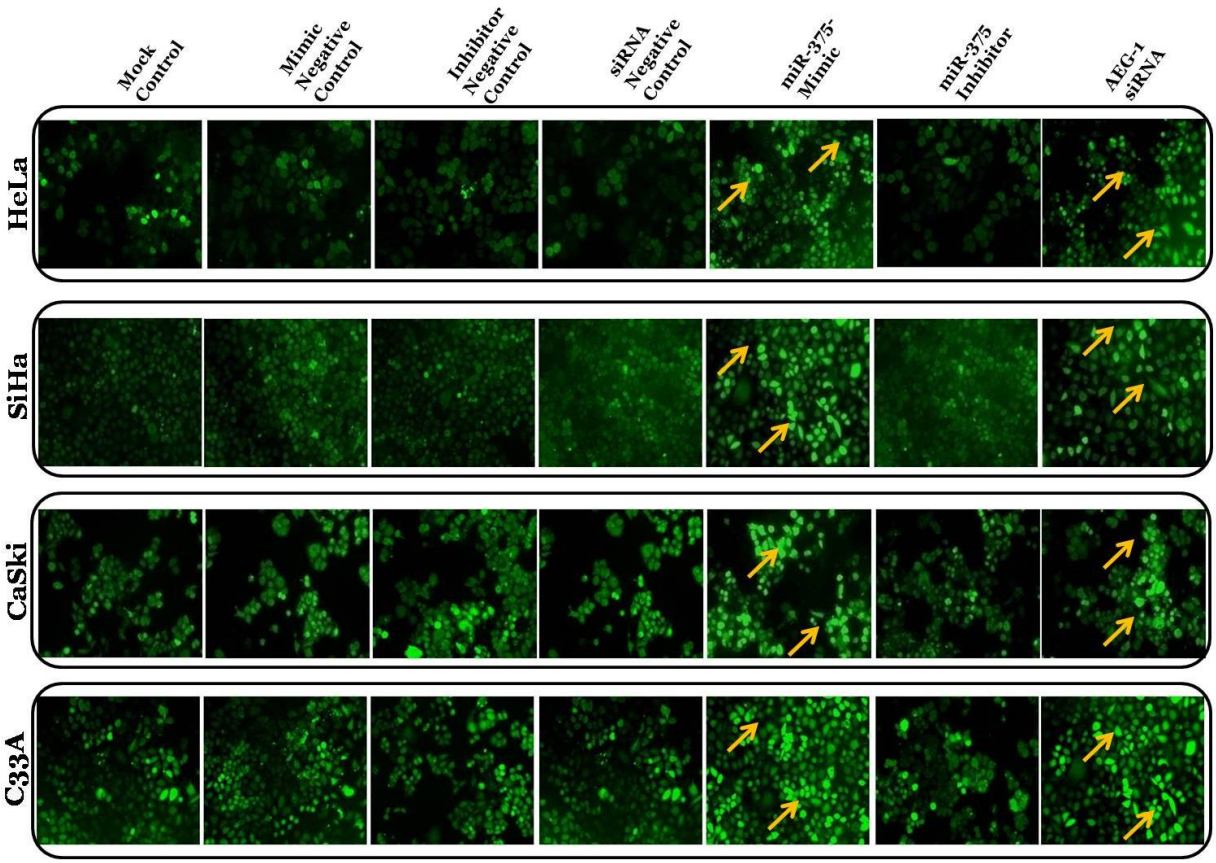


Figure 9. ROS generation in CC cells. Intracellular ROS level was visualized under a Floid cell imaging station (20X). miR-375 transfected cells showed that ROS level was increased when compared to control.

DISCUSSION

Recent evidence has revealed that miRNA act as an oncogene or suppressors by targeting genes involved in survival, apoptosis, metastasis, cell differentiation proliferation, migration and chemoresistance [16-17]. miR-375 is widely present in various tissues or organs and its expression was significantly downregulated in different cancers such as esophageal carcinoma, gastric cancer, glioma and melanoma [18-21]. Therefore, this evidence clearly shows that miR-375 has an important role in regulating CC development and progression. HPV is the main component for causing cervical cancer; however, a comprehensive HPV-miRNA regulatory network in cancer is still largely unknown. In this study, we found that miR-375 was significantly downregulated in squamous cervical carcinoma patients, HPV positive cervical cancer cell lines and HPV 16 positive cervical cancer patients when compared to normal control, which indicated its potential antitumor function. miR-375 plays an important role in the cancer progression. In this research work, by using several microarray data and bioinformatics tools, we identified miR-375 downregulated during HPV infection and AEG-1 as potential target of miR-375. Furthermore, the overexpression of AEG-1 has been identified in different types of human cancers and it contributes to increased cellular migration and invasiveness, proliferation and survival under chemotherapy [22-

23]. From our results, miR-375 downregulated in cervical cancer and target AEG-1. But the molecular mechanism behind the downregulation of miR-375 is still unknown. Therefore, we analysed whether HPV16 plays any role in miR-375 expression. To verify our hypothesis, we further examined HPV 16 regulated miR-375 in CC. Although previous few studies only have been evaluated that potential effects of HPV viral oncogenes on miRNA expression including miR-375 dysregulation. Silencing of HPV E6/E7 oncogenes significantly increased the miR-630 in Ms751 and CaSki cell line. From this results suggested that there may be a negative relationship between miR-630 and HPV E6/E7. Forced over expression of miR-630 could inhibit the invasion and migration properties of CC cells through the inhibition of epithelial–mesenchymal transition. HPV E6/E7 promotes p53 degradation and downregulated miR-630 expression at the transcriptional level [24]. Another study [25] showed that the influence of HPV genes on differential miRNA expressions. HR-HPV 16 is an important risk factor for human head and neck cancer. miR-139-3p downregulation in HPV16 infected cells through the miRNA promoter methylation results to promotes the pro-oncogenic pathway leading to HPV-16 induced head and neck carcinogenesis and miR-139-3p had four direct binding targets to HPV-16 E1 mRNA region. Ectopic expression of miR-139-3p significantly decreased HPV-16 E6-E7 mRNA expressions also resulted in enhancing thep53, p21 tumor suppressor and decreased p21 expressions. Moreover, decreased cell migration, invasion, proliferation and also enhanced cell cycle arrest in G_2-M phase leading to cell death while overexpression of miR-139-3p and also increased sensitization of chemotherapeutic drug cisplatin and 5-fluorouracil to HPV-16 positive cervical cancer cells [25]. Subsequent experiments supported previous *in silico* and experimental analysis identified miRNAs is a major regulator in cancer progression. In this study, we found that miR-375 is one of the most downregulated miRNA in squamous cervical carcinoma patients as well as HPV positive cervical cancer cell lines and also miR-375 mostly downregulated in HPV 16 positive cervical cancer patients when compared to healthy control. miR-375 targets 481 different genes including the potential oncogene AEG-1 and regulates the biological process, molecular function and cellular component. miR-375 targeted genes involved in various biological signaling pathways, particularly in cancer: Angiogenesis pathway, Apoptosis pathway, EGF receptor signaling pathway, Ras pathway and Wnt signaling pathways. Furthermore, our gain-and-loss-of function, ectopic expression of miR-375 and silencing the target AEG-1 induced apoptosis, increased nuclear fragmentation, mitochondrial membrane depolarization and induction of ROS. While, transfecting miR-375 inhibitor, inhibits apoptosis, decreased nuclear fragmentation, mitochondrial membrane depolarization and inhibit ROS generation.

4. CONCLUSION

In conclusion, our integrative *in-silico* analyses and preliminary experimental validation indicated HPV-16 targets miR-375 and contributing to the carcinogenesis of HPV-related CC. Further in-depth

studies would help in understanding the molecular mechanisms beneath the HPV-miRNA regulatory network in cervical cancer.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest concerning this article.

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