Micro RNA biomarker for High risk- HPV 45: omics approach and human sample validation.

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Abstract :

Background:

The most prevalent oncogenic virus is Human papillomavirus (HPV). The mechanism of HPV carcinogenesis is also a fairly straightforward infection mechanism. 95 % of cervical cancers are caused by high-risk HPV infection. Among the rest, HPV-45 is proven to contribute to more than 50% of the cases. cervical cancer is predominantly caused by the high–risk HPV virus. HPV virus

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infection to abnormal cell development to the formation of lesions (by aggregation of abnormal cells), to the spreading of infection inside the cervix, and finally the formation of Cancer is a process of 10 years with the constant assault and prevalence of HPV in the cervix.

Objective :

This study aims to identify miRNA biomarker taegets for early detection of HPV 45.The correlative use of known biomarkers for the prediction of progression is an effective technique. The dysregulation of microRNA (miRNA) is one of the key processes in cancer.

Results :

The MAP kinase pathway and SMAD pathways are the common pathways of HPV 45 induced cancers. The multi-omics approach combined with wet lab techniques help standardise hsa-miR-148-3p suppression as a biomarker for HPV-45.

Conclusion:

Sometimes the body eliminates the HPV virus but the changes induced by the virus in the initiation of the cancer remains. Hence this will be an important marker in the early screening of HPV insuced cervical cancer.

Keywords : Human papillomavirus – 45; biomarker ; microRNA ;OMICS; hsa-miR-148-3p

1. Introduction

The 2020 statistics show that 19.3 million new cases of cancers were identified, while 10 million deaths were reported (Esteller M et.al). The incidence rates for women-related cancer showed high incidence for breast, lung, and cervix cancers (Mathur et al., 2020). Among these 90% of cervical cancer caused by the infection of High-risk Human Papillomavirus (Hr-HPV). Thus, making it the most easily detectable and preventable form of cancer. It is more prevalent in Cervical low- and mid-income countries. Between 84 and 90% of cervical cancer has been reported in low- and middle-income countries namely South Africa, India, China, and Brazil (Hull et al.,2020). Death rates for female breast and cervical cancers, however, were considerably higher in transitioning versus transitioned countries (15.0 vs 12.8 per 100,000 and 12.4 vs 5.2 per 100,000, respectively).

MicroRNAs (miRNAs) are class of small noncoding RNA with 20-25 nucleotides in length

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that modulate the gene expression by partial base pairing with 3′ untranslated region of their messenger RNAs (mRNAs). These small molecules modulates the expression of gene by direct breakdown of targeted mRNA or inhibiting translation. Li Y et.al, reported that altered expression of miRNAs has been reported in carcinomas especially cervical cancer, by altering oncogene expression and tumor suppressor genes thereby causing dysregulation of important intracellular pathways (Li Y et.al,).

Among the DNA Viruses, the HPV virus is known to cause most genital warts and accounts for the major portion (90-95%) of the total cervical cancers. High-risk HPV are those that cause pre and malignant lesions by causing neoplastic changes in the keratinocytes. Low-risk HPV causes benign lesions. HPV 16, 18, 45,32,10,61,2,26,53,7,34,1, 54, etc belong to the group alphaviruses (De Villiers et al ., 2004). Human papillomavirus 45 (HPV45) is a member of the HPV18-related alpha-7 species and accounts for approximately 5% of all cervical cancer cases worldwide.

The role of miRNAs in gene dysregulation has been established in cancer-related studies and hence has given miRNAs a status as a crucial biomarker. Dysregulation of miRNA expression in HPV infection is linked to a vast extent to E5, E6, and E7, viral oncogenes. Since miRNAs are easy to detect, fast, sensitive, and accurate this makes it a good candidate for biomarker. Eventhough many research are conducted there still exist a wide lacunae in finding the association of HPV45 variants with cervical cancer risk. So, this study is done to assess the miRNA as biomarker for High risk HPV- HPV 45

2. Materials and Methods :

This study was done in south Indian state of Tamil Nadu. Ethical consent was obtained from the institutional ethical committee of Hycare Hospitals, Chennai. The study was conducted on the samples sent for testing to Regenix Super Specialty Laboratories Pvt.Ltd., Chennai from its branches all over Tamil Nadu for pap smear and HPV molecular testing through LBC samples, and the excess samples after testing were used for this study, and hence the higher rate of positivity. Consent and data were collected from the patients. The patients in the study group were aged between 21 -68 years with the mean age of the population being 38 years. The mean age for positivity in this study was found to be 45 years .

confirmed with PCR technique through a CE-approved market kit.

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Ethical approval was obtained and consent and patient history were collected from the patients. The samples received in Regenix Super Specialty laboratories for LBC and /or HPV testing and tested positive for HPV 16 or HPV 18 were used for the study.

- Ethical approval for the project was obtained from HYCARE Wounds IEC

  Project no:027/HYC/IEC/2018 dated:13.12.2018

- Ethical approval for the project was obtained from HYCARE Super specialty Hospital IEC

  Project no: 027 /HSSH-EC/2022 dated: 04.02.2022

HCK1T cell lines are primary cervical epithelial cells. ATCC product code is PCS-480-011. The subculturing and passage were done according to the ATCC instructions (www.atcc.org.). The doubling time is 24-48 hours.

Reagents and materials

- ❖ 75 cm^2 cell culture flask.
- ❖ Cervical Epithelial Cell Basal Medium (ATCC PCS-480-032)
- ❖ Cervical Epithelial Cell Growth Kit (ATCC PCS-480-042)
- ❖ Trypsin
- ❖ EDTA solution
- ❖ DMSO (for preservation)
- ❖ Co_2 incubator

Procedure:

- ❖ Passage was performed at 85-90% confluence of cells.
- ❖ The growth media was mixed and warmed to 37 °C.
- ❖ Warmed Trypsin was added to the flasks after the removal of media and DPBS solution wash. Gentle agitation was given and incubated for 3- 5 minutes.
- ❖ The cells were observed under the microscope for detachment from the flask.
- ❖ Trypsin neutralizing solution was added to the flasks and swirled.
- ❖ The disassociated cells were transferred to a centrifuge tube and centrifuged to 1000 rpm for 5 minutes. The neutralizing solution was aspirated and the cells re-

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suspended in growth media.

- The cells were transferred to a fresh flask.
  - C-33A cell lines are carcinoma cells of primary cervical epithelial cells. ATCC product code is HTB-31. These are HPV-negative cancer cell lines. The subculturing and passage were done according to the ATCC instructions (www.atcc.org.). The doubling time is 1.36 days.

Reagents and materials

- 75 cm^2 cell culture flask.
- ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003.
- fetal bovine serum to a final concentration of 10%.
- Trypsin
- EDTA solution
- DMSO (for preservation)
- Co_2 incubator

Procedure:

- The cell layer was rinsed with 0.25% (W/v) of trypsin-EDTA solution after the culture media was removed.
- Incubation of 5-15 minutes was carried out and complete dispersion of cell layers was observed.
- The dispersed cells were collected using 6-8 ml of media resuspended into a new culture flask and incubated at 37 °C for growth.
- Transfection Studies for HPV 45- HCK1T was transfected with E6 and E7 genes and expression studies were carried out according to the protocol of Kim and Eberwine ., 2010 using Thermo- scientific viral transfection kit - AAV-MAX.

HPV-45 E6 and E7 genes (plasmids) were transfected into the HCK1T cell lines .

Adenoviral, on coretroviral, and lentiviral vectors have been used extensively for gene delivery in mammalian cell culture and *in vivo*

Reagents and Materials

- HPV 45 (E6 and E7) plasmids
- HCK1T cell lines

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- ❖ AAV-MAX Transfection Reagent\
- ❖ AAV-MAX Transfection Booster
- ❖ AAV-MAX Enhancer

Procedure:

- ❖ The cells are cultured and passaged.
- ❖ The transfection protocol of the kit was followed for transfection.
- ❖ The cells were assayed and checked for transfection.

Western blot studies for differential protein expression analysis

SDS and western blot analysis were carried out to understand the different proteins and pathways expressed by the cancer cell lines.SDS and Western blot analysis were carried out using Biorad western blot kit – Catalog no.6376 following the kit instructions. (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6376.pdf).

OMICS studies for identification of miRNA

The Data sets and pathway analysis was carried out to understand the expressed pathways in cervical cancer. This data was combined with the western blot analysis to determine the increased and decreased expression of proteins.

The MAPK, SMAD, ERK1/2, and PI3K showed predominant differential expressions in cervical cancer. The mRNA and miRNA involved in this pathway were elucidated through expression studies (Heier et al., 2020)

The antibodies used for western blot are:

- MAPK antibody -Anti-p38 MAPK antibody [HL1006] ab308333
- ERK1/2 antibody - Anti-ERK1 + ERK2 antibody [EPR17526] ab184699
- SMAD antibody -Anti-SMAD 1/5/8/9 antibody ab13723
- PI3K antibody-Anti-PI 3 Kinase catalytic subunit gamma/PI3K- gamma antibody [EPR25156-60] ab302958

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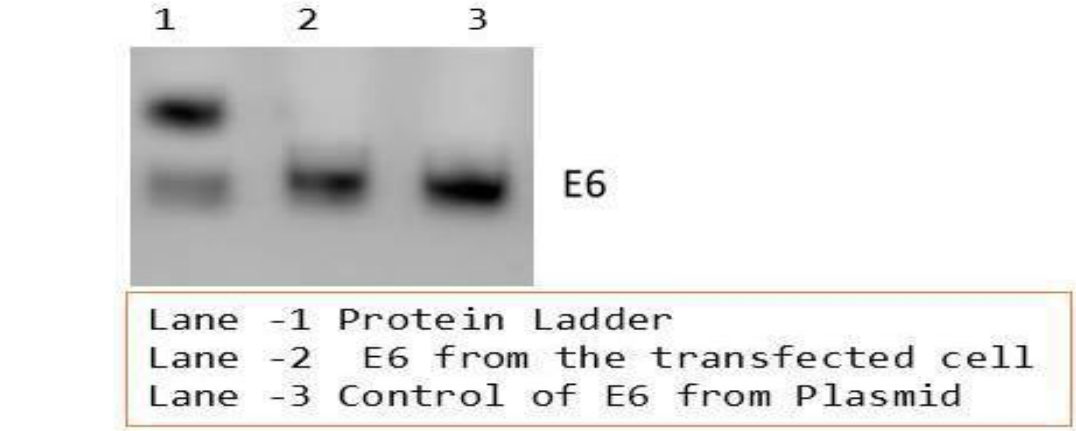
3. Results

Figure 1: Western Blot analysis of confirmation of transfection in HCK1T cell line



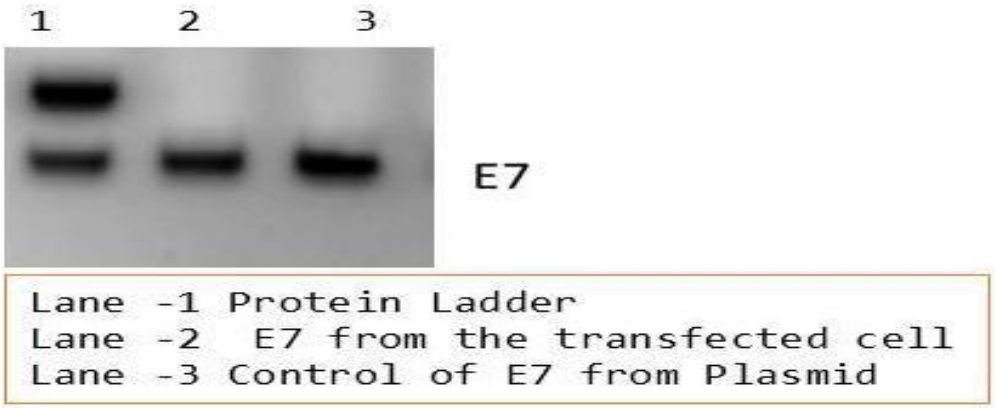


Figure 2: Western Blot analysis of confirmation of transfection in HCK1T cell line

HPV 45 E6 and E7 plasmids were transfected in the HCK1T (figure 2) cell line and after 2 passages western blot analysis was carried out to confirm the transfection. Hence for the HPV 45 transfection was carried out by these two early proteins. The same was confirmed with the pathway analysis of the transfected cells and normal cells as shown in figure 3.

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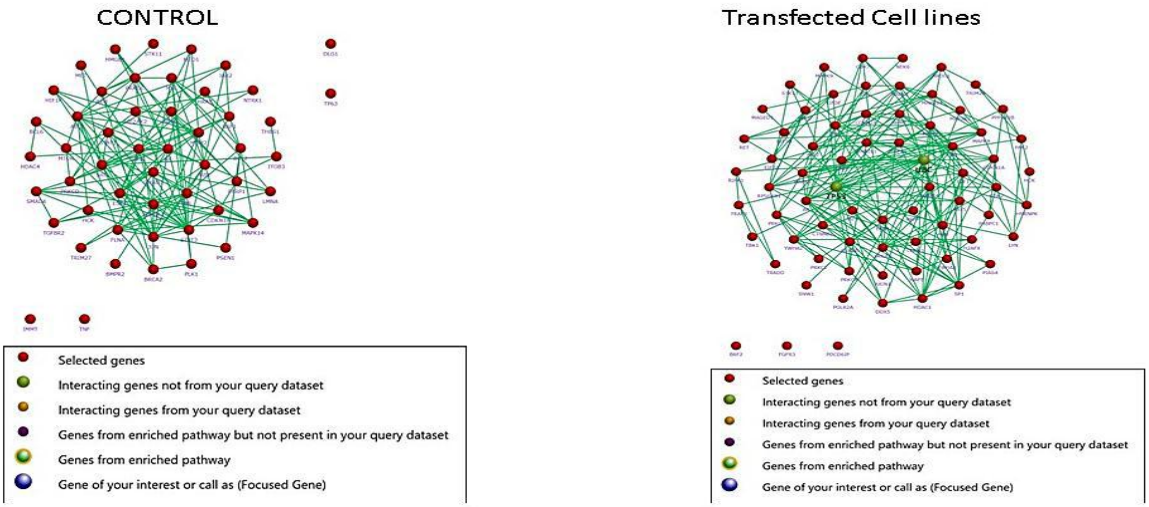
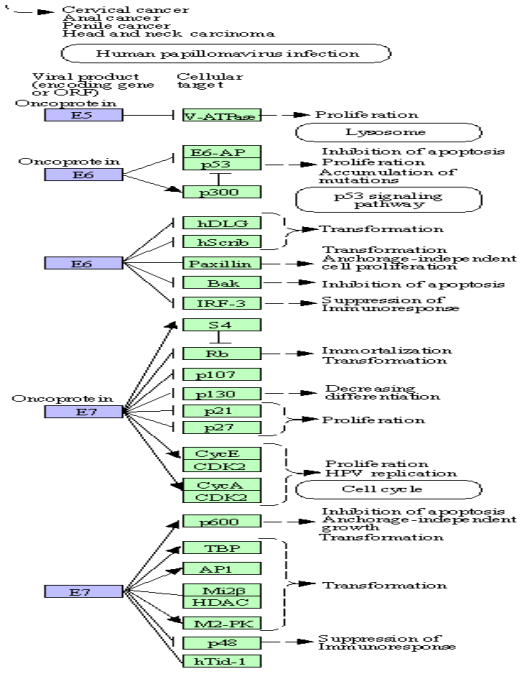
Figure 3: Pathway analysis confirmation of transfection



This analysis showed the efficiency of transfection. It was seen that there were significantly more genes expressed in the transfected cell line. Hence, this transfected cell line was used for further analysis of HPV 45.

Figure 4: KEGG analysis of HPV viral proteins



KEGG pathway analysis was carried out to learn about the different pathways and proteins carried out in cervical cancer (figure 4). These pathway enrichment analysis results

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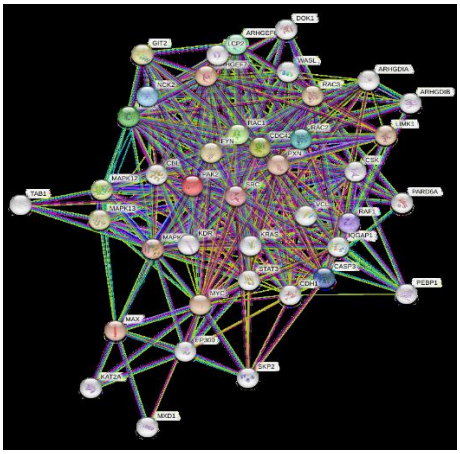
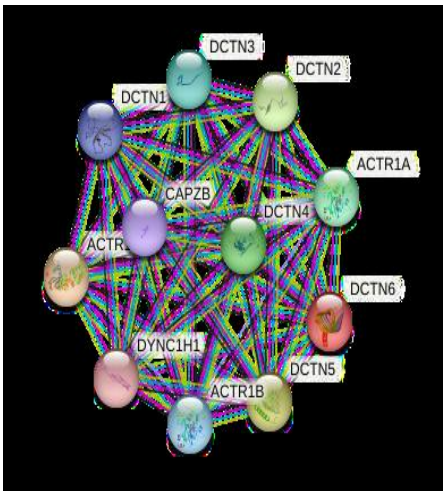
formed the basis of the investigations to confirm the proteins expressed by the cell lines through western blot analysis. One of the predominant pathways seen in the Kegg analysis was the blocking of the p53 and pRb genes by the E6 and E7 proteins. Apart from this PI3K/Akt, ERK/MAPK, JAK-STAT, NF-κB, and Wnt/ β-catenin were some of the predominant pathways in cervical cancer. Thus in HPV 45 DCTN6 and P16 pathways were expressed.

Figure 5: HPV 45 Pathway enrichment analysis (a. All pathways, b.DCTN6 pathway, c. p 16 pathway).



a. HPV45 Pathway Analysis



b. HPV45 DCTN6 Pathway Analysis

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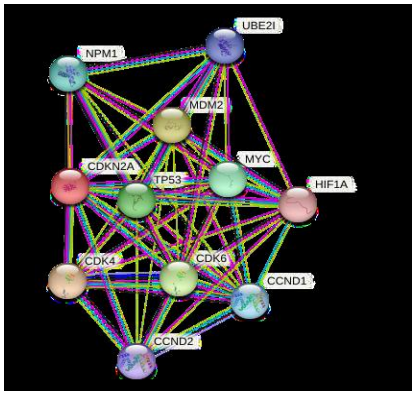
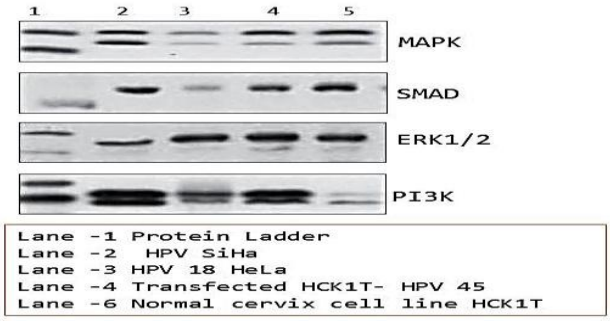


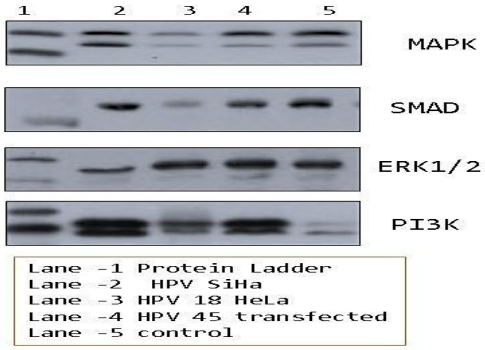
c. HPV45 p16 Pathway Analysis

Figure 6: Western blot analysis of proteins expressed in cell lines based on the important pathways in cervical cancer



Western blot analysis , figure 6, showed that the above cell lines showed differential protein expression patterns in MAPK, SMAD, ERK1/2, and PI3K. The pathway enrichment analysis was carried out to understand the pathways used by HPV 45 (figure 5).

Figure 7: mRNA expression relative to pathways



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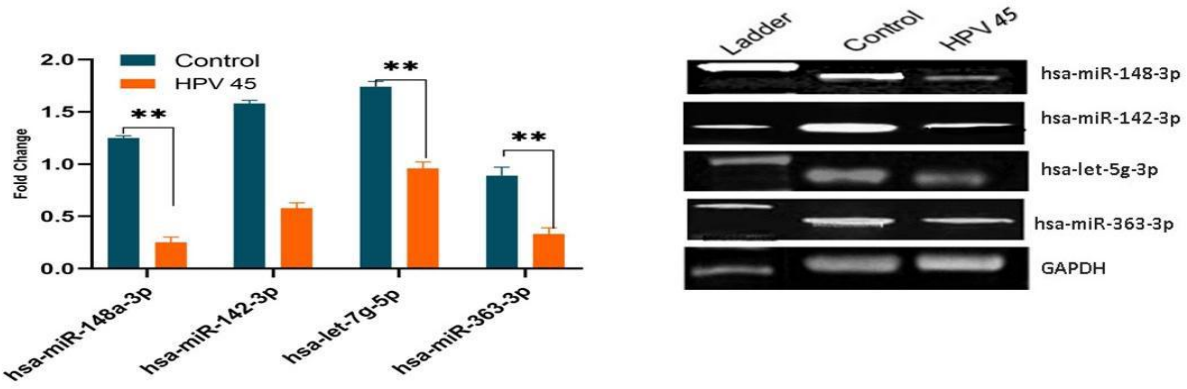
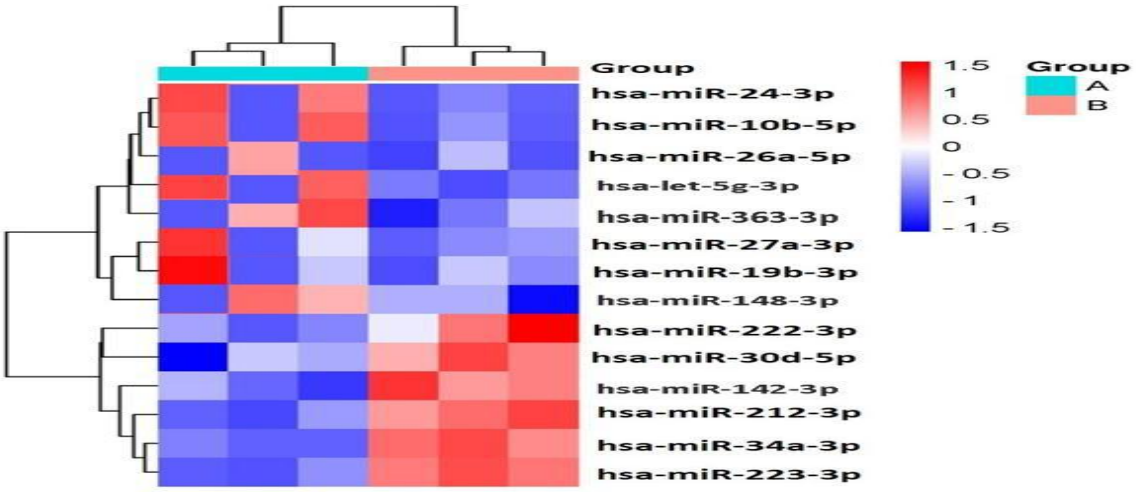
The dysregulation of mRNA (figure 7) and consequently miRNA plays a significant role in cancer. The genes responsible for the protein expression were analyzed. As compared to controls there is upregulation in the genes that encode for the pathway triggering proteins in the Siha cells. The expression of the MAPK and SMAD distinguishes the HPV45 transfected cell line from the HPV 18 HeLa cell line. The results are consonant with that of the protein expression. The results were concordant with the protein study.

Figure 8: HPV 45 miRNA from cell lines



The quantification of miRNA based on the above studies showed the expression of 4 miRNAs from cell culture (in vitro), namely hsa -let -7a-5p, hsa -let -7b-5p, hsa -miR -98-5p and hsa-let-7f-5p. This miRNA showed increased expression in the cell line studies (figure 8). The results of expression were concordant with that of cell culture screening, showing elevated expression in the positive samples. Known positive samples were used for validation (figure 9).

Figure 9: Confirmation of miRNA with human samples HPV 45



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Discussion and Conclusion:

HPV45 is reportedly more common in adenocarcinoma than in squamous cell carcinoma of the cervix. Roughly 5% of cervical cancers are positive for HPV45 globally, while this proportion was described to vary from 3% in Eastern Asia to 9% in Africa. The present study was done in south Indian state of Tamil Nadu. The patients in the study group were aged between 21 - 68 years with the mean age of the population being 38 years. The mean age for positivity in this study was found to be 45 years.

HPV-45 is the third most frequent HPV type in cervical carcinoma and adenocarcinoma. Tjalma WA et.al, reported that women less than 30 years have twice (4%) as many invasive cervical cancers related to HPV-45 than other types. Cora Ngelange et.al, reported that among HPV types other than types 16 and 18, the associations of HPV with risk of squamous cell carcinoma were strong est for HPV45.

A biomarker panel for genotype identification was developed through invitro screening and confirmed through testing in positive samples. miRNA biomarkers were validated for HPV 45. That was seen to be statistically the highest in the Indian population. In the present study, out of all subjects, 3 subjects were tested for HPV 45 and all were found to be positive. In this study patients with diabetes mellitus and hypertension seemed to have a higher incidence of HPV Positivity.

Pap smear results showed the incidence of inflammation to be highest among the group studied. HPV positivity and inflammation were positively correlated. Inflammation, erosion, chronic cervicitis, and hardening were the predominant conditions of the cervix and they were positively correlated with a positive HPV test. Symptoms such as intermittent bleeding, foul-smelling discharge, and colored discharges were seen as red flags for HPV positivity.

Okoye et al. found that when miRNAs are analyzed in both serum and cervical samples, they may have a better association with malignancy, and therefore, can distinguish abnormal cervical lesions. Nascimento NP et.al, found that thirty three microRNAs for HPV 45 were

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evaluated in the eligible studies and 17 showed up-regulation in women with precursor lesion and cervical cancer and 16 microRNAs showed decreased expression in these same groups of women compared to healthy controls.

In this study, SDS page analysis showed marked difference in the expression of protein due to the alteration in HPV 45. HPV 45 has 70% genes similar to HPV 18. Since the number of samples was lesser in HPV 45, confirmation requires more samples. But the data obtained in the present study were consistent with the invitro studies.

5. Conclusion

Therefore, defining promising circulating miRNAs or specific miRNA signatures of biological fluid samples can beneficial for the screening, diagnosis, prognosis and clinical monitoring of women with cervical cancer

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