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COMPARISON OF HUMANPAPILLOMAVIRUS MESSANGER(E6)RNA EXPRESSION AND DNA AS BIOMARKER IN SCREENING OF VIAVILI POSITIVE CASES IN TERTIARY CARE

Pathology

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ABSTRACT

Cervical cancer is second most frequent cancer of women in the world. HPV infection is now a well established cause of cervical cancer . Currently used screening programmes are detecting cases in which only 15-20 percent of women found to have an abnormality significant enough to need treatment, other 80-85 percent were probably false alarms. The oncogenic process in cervical cancer is initiated and mediated by upregulation of E6/E7 mRNA proteins and their over expression. So HPV E6 mRNA detection as a triage test could actually detect a neoplastic transformation and may help to reduce the false alarms.**AIMS AND OBJECTIVES :**To compare the expression of HPV E6 mRNA verses the HPV DNA as a biomarker in the screening of cervical cancer .Biopsy is used as gold standard for screening.**MATERIALSAND METHODS:** This a prospective study conducted in Institute of Social Obstetrics and Govt. Kasturba Gandhi hospital, Madras medical college, 41 cases positive for VIA/VILI in colposcopy were selected and subjected to conventional cytology and cervical biopsy ,part of biopsy was collected in RNA later solution and were typed for HPV DNA 16& 18,HPV E6mRNA Expression.the results of above test are compared with histopathology as gold standard. **RESULTS:** The specificity of E6 mRNA was 100% for both high grade and low grade lesions.But the sensitivity was less than HPV DNA and cytology for both low and high grade lesions.With increasing severity of lesion expression of E6 mRNA was found to increase.**CONCLUSION:** Low positivity rate and the high specificity makes E6 mRNA better biomarker in a screening of cervical cancer.

KEYWORDS

cervical cancer, cytology,HPV DNA,HPV E6mRNA,.

INTRODUCTION:

The commonest cancer cause of death among women in developing countries is cervical cancer.[1] In TamilNadu northeastern districts have a high incidence of cervical cancer which is attributed to infection with human papilloma virus.[2] These regions show high prevalence of HIV infection too. South and southeast Asian countries have high burden of cervical cancer which is due to the high prevalence of HPV infection and inadequate screening[1]And it is the second most common cancer of women in reproductive age group Carcinoma cervix is unique among human cancers because it is the first carcinoma found to be directly attributable to the effects of infectious agent. This association was first discovered by Dr.Heraldzurhausen. He was awarded Nobel prize in medicine in 2008 for that.HPV belong to the family of Papillomaviridae[10] they are epitheliotropic virus. Infection with high-risk HPV is the main cause of cervical intraepithelial and invasive neoplasia and HPV DNA has been detected in >90% of cervical carcinomas, with the most common HPV types identified as HPV 16, 18, 31, 33,and 45 .[3-6] HPV is a common virus among women, particularly in younger age groups, and most infections are transient and asymptomatic . Patients with persistent infection with these HPV types have a clearly enhanced risk of developing cervical carcinoma .

Large-scale screening studies have shown HPV testing as more sensitive than cytology for the detection of high-grade cervical lesions .[7-10] However, low specificity of current assays and commercial kits hampers the use of HPV testing in screening. The combination of HPV DNA detection and cytology is more suitable for risk assessment of progression to cervical intraepithelial neoplasia (CIN) grade 3 and carcinoma than cytology alone .[11,12]Viral infection is targeted at the parabasal keratinocytes, but with a relatively low level of viral E6/E7mRNAexpression[19].However, in the upper spinous and granular layers of the epithelium of squamous intraepithelial lesion (SIL) and cervical carcinoma, an increasing expression of the viral oncogenes E6 and E7 occurs. This expression is necessary for conversion to and maintenance of malignancy [13-15], and hence the detection of the E6/E7 transcripts of high-risk HPV types might serve as a better risk evaluation factor than mere DNA detection for the development of HSIL.The same would be true for the progression to cervical carcinoma .[16]

AIM OF THE STUDY:

The aim of the present study was to compare the detection of HPVmRNA from the carcinogenic HPV types 16 and18 with the detection of HPV DNA in an outpatient screening population with VIA VILI positive.

OBJECTIVES:

To assess the expression of HPV DNA in cervical biopsy samples. To assess the expression of HPV E6 mRNA in the cervical biopsy samples. To correlate and compare the expression of HPV E6 mRNA with conventional cytology, HPV DNA and cervical biopsy.

MATERIALS AND METHODS:

This is a prospective study conducted at the Institute of social obstetrics and Kasturba Gandhi hospital for women and children, Madras medical college .

This study involved 41 women who have been diagnosed with positive VIA / VILI in colposcopy are subjected to conventional cytology and cervical punch biopsy. The part of the biopsy sample is collected in RNA Later solution for HPV DNA testing and HPV E6 mRNA expression. The results of above test are compared with histopathology as gold standard.

INCLUSION CRITERIA:

1. Women in the age group of 21-65
2. Non pregnant women
3. VIA/VILI Positive
4. Women without prior treatment for cervical pre-neoplastic and neoplastic lesions

EXCLUSION CRITERIA:

1. Pregnant women
2. Menstruating women
3. Intrauterine Device users
4. Women who have undergone prior treatment for cervical neoplastic and pre-neoplastic lesions

After obtaining informed consent, detailed history was obtained. After complete physical examination, specimens- cervical scrapings and cervical punch biopsy were collected.

Conventional PAP smear:

To collect samples for conventional PAP,Ayers spatula was inserted in to cervix and gently rotated at 360 degree and sample is smeared into a grease free slide and fixed in alcohol. After fixation, smear was stained with PAP stain . The PAP smears were examined and reported according to Bethesda system of classification.

Cervical biopsy:

Cervical biopsy were taken using punch biopsy forceps and put in 10% neutral buffered formalin and sent for Histopathological examination.

After histopathologic processing, the tissue sections were stained with haematoxylin and eosin .

The reports are given as
- Normal
- Chronic non-specific cervicitis
- CIN I
- CIN II
- CIN III
- Invasive squamous cell carcinoma
- Well differentiated
- Moderately differentiated
- Poorly differentiated
- Adenocarcinoma

HPV DNA Typing and HPV E6 mRNA Expression:
The tissues were collected in RNA later solution and transported by cold storage to laboratory and kept at 4^0c for overnight, the excess RNA later solution drained and tissues were frozen at -80^0C.

DNA Isolation from tissues:
Tissues were washed thrice with Phosphate buffer saline (PBS) buffer. Approximately 30 mg of tissue were minced and homogenized in tissue lysis buffer. Proteinase K was added to the homogenate and incubated at 37°C overnight. After incubation, equal volume of P:C:I (Phenol/ chloroform/ isoamylalcolhol) was added to the lysate and centrifuged at 10,000 rpm at 25°C for 25 min. The aqueous phase was transferred into a fresh tube and 2.5 volume of 100% ethanol was added to it to precipitate DNA. DNA pellet was transferred to Eppendorf tube, containing 70% Ethanol and centrifuged at 10,000 rpm for 15 min at 4°C. DNA pellet was air dried and dissolved in appropriate volume of 1X Tris EDTA.

RNA isolation from tissue:
Total RNA was isolated from the tissue samples by RNeasy mini kit (Qiagen®) as per manufacturer's protocol. The RNA samples were quantified using Nano drop (Thermo Scientific, USA) and the integrity of the RNA samples were checked by 1 % agarose gel electrophoresis and stored at -80°C until further use.

cDNA conversion:
A 20 µl volume containing 5 µg of total RNA, 1x buffer, 100 µM random primers, 0.1M DTT, 100mM dNTP and 200 Units of Superscript III (Invitrogen, USA) was used for cDNA synthesis, with the following thermal conditions: RNA was pre incubated with random hexamers for 20min at 65˚C followed by 90 min at 50˚C and 15 min at 72˚C. Further, a 1:25 dilution was prepared from the 20 µl stock cDNA and stored at -20°C.

Polymerase Chain Reaction (PCR):
Polymerase Chain reaction was performed to find out the presence of HPV 16 and 18 in cervical cancer tissue samples and β-Globin gene was used as positive control. PCR was carried out in a total volume of 10 µL using 1X PCR buffer, 1.5 mM $MgCl_2$, 100 µM dNTPs, 60 nM each of HPV16 and HPV18 specific forward and reverse primers, 0.5 Units of Taq polymerase and 100ng/Rxn tissue DNA. The thermal cycling conditions were 94°C for 10 min; 40 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec followed 72°C for 7 min and 4°C hold. The primers used for the analysis are HPV 16 E6 Fwd - 5' CTGCAATGTTTCAGGACCCA 3', HPV 16 E6 Rvs-5' TCATGTATAGTTGTTTGC AGCTCTGT 3'; HPV 18 Fwd - 5' AAACCGTTGAATCCAGCAGAA 3', HPV 18 Rvs -5' GTCGTTCCTGTCGTGCTCG 3' and β-Globin Fwd - 5' GGATCTGTCCACTCCTGATGCTG 3', Rvs - 5' TCACTCAGTGTGGCAAAGGTGC 3'.

Expression analysis:
Expression of HPV 16 E6 mRNA was quantified by Custom designed Taqman Assay using ABI Quantstudio6 with primers specifically amplifying HPV16 and 18 E6. PCR reaction was carried in a total volume of 10 µl containing 0.5 µl of 20X Primer-Probe mix (1X), 5 µl of 2X Universal Master Mix (1X), Applied Biosystem, USA and 1 µl of 1:25 cDNA with 3.5 µl H_2O. Thermal protocol followed was: 50°C for 2 min, 95°C for 10min, 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

The presence of HPV DNA 16 and 18 is reported as present or absent.The results of HPV E6 mRNA expression is given as amplification plot ,which is then computed to numerical expression with special software.

STASTISTICAL ANALYSIS
The information collected from various screening methods like cytology, HPV DNA and HPV E6 mRNA were recorded .Statistical analysis were done with SPSS 20 version. Comparative analysis were done with Chi-Square test and P values less than 0.05 were considered statistically significant.

The P value was calculated for conventional cytology, HPV DNA, HPV E6 mRNA in cervical non- neoplastic, preneoplastic and neoplastic lesions, showing their statistical significance in correlation with biopsy. The sensitivity, specificity, positive predictive value and negative predictive value were also calculated and they were compared with other similar methods.

OBSERVATION AND RESULTS
The 41 VIAVILI Positive cases are randomly selected from patients attending colposcopy department at the Institute of social obstetrics and Kasturba Gandhi hospital for women and children,Of these 25 were Low grade lesions,5 were High grade lesions and 11 were invasive carcinomas (TABLE 1).

Table 1:Distribution Of VIA/VILI Diagnosis In The Study Group

n=41

VIA/VILI	LGL	HGL	INV CA
FREQUENCY	25	5	11
PERCENTAGE	61%	12.2%	26.8%

These 41 patients were subjected to conventional cytology cervical biopsy, HPVE6mRNA assay. The biopsy report was confirmative and taken as gold standard. Of the 41 VIAVILI positive cases biopsy turned out to be chronic cervicitis -10(24.4%),CIN I-15(36.6%),CIN II-3(7.3%),CIN III-2 (4.9%).SCC-11(26.8%)(TABLE 2).

Table 2: Age Wise Distribution Of Biopsy In The Study Group
n=41, % with in age group

BIOPSY	AGE IN YEARS				
	21-30	31-40	41-50	51-60	61-70
CERVICITIS	1(30%)	4(28.6%)	2(28.6%)	2(30%)	1(16.7%)
CIN I	3(70%)	5(35.3%)	3(39.7%)	2(30%)	2(33.3%)
CIN II	0	2(11.1%)	1(10.3%)	0	0
CIN III	0	2(11.1%)	0	0	0
SCC	0	1(14.0%)	3(21.4%)	4(40%)	3(50%)

Majority of cervical non neoplastic and preneoplastic lesions were commonly seen in reproductive age group women in the study group.

Table 3: Cytology Results

n=41

CYTOLOGY	Frequency	Percentage (%)
ASCUS	1	2.4
HSIL	3	7.3
LSIL	18	43.9
NIL	8	19.51
POSITIVE FOR MALINGNANCY	11	26.8
Total	41	100.0

Of the 25 low grade lesion in VIA/VILI, 7 were reported as NIL,1 was reported as ASCUS, 17 reported as LSIL. Of the 5 high grade lesions 1 was reported as NIL, 1 reported as LSIL and 3 reported as HSIL. All the invasive carcinoma reported as SCC in cytology(Table 3). Concordance of VIA/VILI and cytology was 100% in invasive a carcinomas only.(Table 4).

The 8 NIL cases turned out to be cervicitis in biopy,18 LSIL cases 14 turned out to be CIN I,1 to be cervicitis, 1 to be CIN III,2 to be CIN II.1 ASCUS turned out to be CIN I,3 of the HSIL turned out to be CIN II&CIN III and all the 11 POSITIVE FOR MALIGNANCY turned out to be SCC only. The concordance of cytology with biopsy 100% in SCC and HSIL, but in LSIL it was less. (TABLE 5).

Table 4:Comparison Of Cytology With VIA/VILI

n=41

VIA/VILI	CYTOLOGY				
	NIL	ASCUS	LSIL	HSIL	SCC

LGL=25	7(77.8%)	1(100%)	17(94.4%)	0	0
HGL=5	1(22.2%)	0	1(5.6%)	3(100%)	0
INVASIVE CA=11	0	0	0	0	11(100%)

Pearson Chi-Square=57.400* P<0.001

Table 5:Comparison Of Cytology With Biopsy

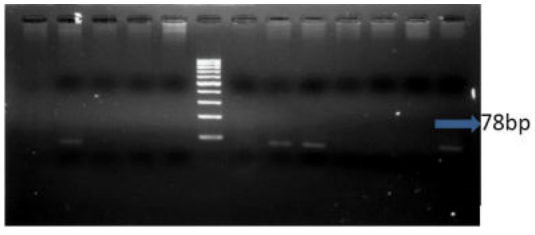
n=41

LBC	BIOPSY DIAGNOSIS				
	CERVICITIS	CIN I	CIN II	CIN III	SCC
NIL	8(90.%)	0	0	0	0
ASCUS	0	1(6.7%)	0	0	0
LSIL	1(10%)	14(93.3%)	2(66.7%)	1(25%)	0
HSIL	0	0	1(33.3%)	2(75%)	0
SCC	0	0	0	0	11(100%)

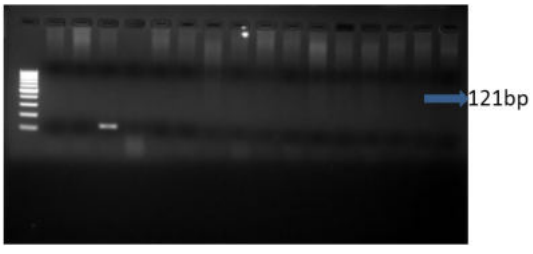
Pearson Chi-Square=90.883* P<0.001

HPV DNA RESULT

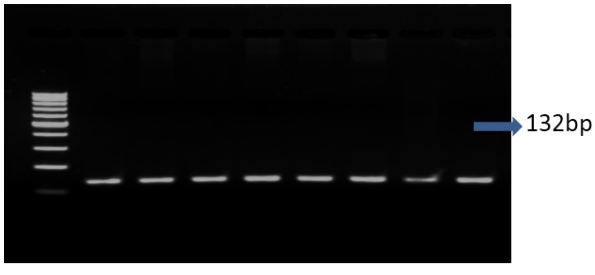
Presence of HPV16 & HPV18 E6 DNA in Cervical cancer samples: Presence of HPV DNA in cervical cancer tissue was confirmed by PCR reaction. Below shown are the representative gel pictures of HPV16 E6, HPV18 E6 and β-Globin amplification respectively.



a)Amplicon of HPV16 E6 DNA in Lane 2, 8,9,13.Lane 6: 100 bp ladder



b)Amplicon of HPV18 E6 DNA in Lane 3. Lane 1: 100 bp ladder



c) Amplicon of β-Globin in Lane 2-9. Lane 1: 100 bp ladder

Among 41 cervical cancer samples, 18 samples were HPV 16 positive and 2 was HPV 18 positive. Percentage of samples positive for HPV DNA 16 and 18 was 48.78%.Among the 20 cases which are positive HPV 16 was found to be positive in 80% Of cases, Only 20 % of cases showed positivity for HPV 18 .HPV 18 Positive cases were found to be CIN I in biopsy.HPV 16 was positive in 1 cervicitis,5 CIN I,2 cases of CIN II and CIN III,10 of SCC (TABLE 6).

Table 6: Distribution Of HPV DNA 16 &18 In Relation To Biopsy

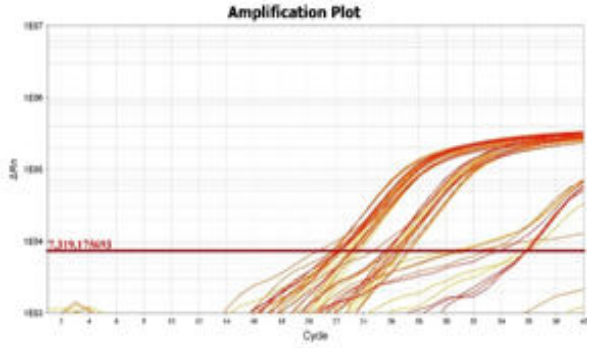
HPV 16&18	BIOPSY DIAGNOSIS					
	CERVICITIS	CIN I	CIN II	CIN III	SCC	
POSITIVE	1(5.6%)	5(16.6%)	2(11.1%)	2(11.1%)	10(55.6%)	20
NEGATIVE	9(39.9%)	10(51.5%)	1(4.3%)	0	1(4.3%)	21
TOTAL	10(24.4%)	15(36.6%)	3(7.3%)	2(4.9%)	11(26.8%)	41

Pearson Chi-Square = 21.203* p<0.001

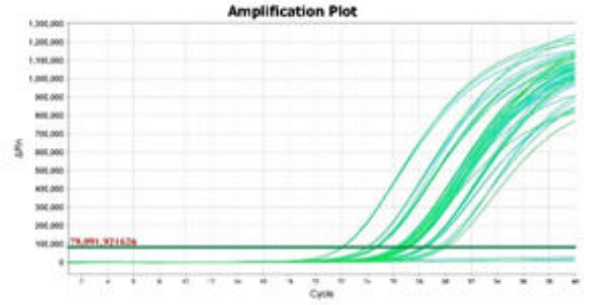
HPV E6 mRNA expression RESULTS:

Below are the amplification plots showing amplification curve of HPV16 E6 mRNA and GAPDH (glyceraldehyde 3 phosphate dehrogenase) (Reference gene).

AMPLIFICATION PLOT FOR E6 mRNA



AMPLIFICATION PLOT FOR GAPDH



These graphical values are converted to numerical values with special software.

Among 10,chronic cervicitis case HPV E6 mRNA was positive in 1,5 0f CIN I cases were positive,1 0f both CIN II and CIN III was positive, except one case of SCC all Were positive for HPV E6 mRNA. (TABLE7)

Table7: Distribution Of HPV E6 mRNA Expression In Relation With Biopsy

n=41

HPV E6 mRNA	BIOPSY DIAGNOSIS				TOTAL
	CERVICITIS	CIN I	CIN II&III	SCC	
POSITIVE	1(10%)	5(33.3%)	2(40.0%)	10(90.9%)	18(43.9%)
NEGATIVE	9(90%)	10 (66.7%)	3(60.0%)	1(9.1%)	23(56.1%)
TOTAL	10	15	5	11	41

Table 8: Comparison Of Sensitivity, Specificity, PPV and NPV In CIN 1 Cases, For Cytology, HPV DNA, and HPV E6 mRNA

CINI	CYTOLOGY IN %	HPV DNA IN%	HPV E6 mRNA IN%
SENSTIVITY	93.33	33.33	33.3
SPECIFICITY	92.31	96.15	100
PPV	87.50	83.33	100
NPV	96.	71.43	72.22

Table 9: Comparison Of Senstivity, Specificity, PPV and NPV For CIN II & III

CASES FOR CYTOLOGY,HPV DNA,HPV E6 mRNA

CIN II&III	CYTOLOGY IN %	HPV DNA IN%	HPV E6 mRNA IN %
SENSTIVITY	60	80	40%
SPECIFICITY	94.44	97.2	100%
PPV	60	80	100%
NPV	94.44	94.59	92.31%

Table 10: Comparison Of Sensitvity, Specificity, PPV and NPV In Cytology, HPV DNA, HPV E6 mRNA In SCC Cases

SCC	CYTOLOGY IN %	HPV DNA IN %	HPV E6 MRNA IN %
SENSTIVITY	100	90.91	90.91
SPECIFICITY	100	100	100
PPV	100	100	100
NPV	100	96.77	96.77

DISCUSSION:

Currently we have three main modalities of cervical cancer screening which includes cytology based, visual based and HPV detection.

Visual based methods are like VIA/VILI are integrated in to the primary health service. The feasibility of VIA/VILI depend on training and monitoring. There are also concerns about its reproducibility and quality controls of VIA/VILI in field trials.

Cytology has limitations as high rate of false negative results due to variable lesion distribution in the cervix,exfoliative variability in cell samples, smear preparation and staining, microscopic reading and variation in the readers stringency in reporting[17]. Several metaanalysis have reported low pap smear sensitivities-with a median of 50%[18,19]. The sensitivity further falls for post-menopausal women due to physiologic changes of cervix.

Co-testing with HPV PCR was introduced. But this lacks specificity because most of the HPV infections are transient.[20,21&22]So to be even more specific now they are studies going on in HPV E6/E7 mRNA assays. Progression to malignancy requires over expression of E6/E7 in the integrated HPV genomes. It follows that E6 transcripts could be useful marker of disease progression. In our study we have tested this possibility; using data's from cytological, histological, HPV DNA testing and compared with E6 mRNA assay.Histopathological results were taken as gold standard for comparison.

This was a prospective study involving 41 patients. 41 VIA/VILI positive cases were randomly selected from the patients attending the colposcopy department.

Of the 41 VIA/VILI positive cases 25 were low-grade lesion,5 were high grade lesion,11 were invasive carcinomas.

The percentage of concordance with biopsy was 60% for LGL, 60% for HGL, and 100% for SCC in the study group. Thus sensitivity of VIA/VILI is almost similar for both LGL and HGL.

Of the 41 cases, chronic cervicitis was found equally (28.6%) in age group 31-40 and 41-50. Majority of CIN I lesion(38.6%) were in age group 31-40,CIN II and CIN III were also common in age group 31-40. All SCC were common in age group 41-60.Majority of cervicitis and CIN(80%) were found in reproductive age group women. Majority of SCC(80%) found in post-menopausal women.

CERVICAL CYTOLOGY

In cervical cytology reports of 41 cases,8 were given as negative for intraepithelial neoplasia (NIL), 18 were given as low grade Squamous intraepithelial lesion (LSIL), 3 were given as high grade squamous intraepithelial lesion (HSIL), 1 as ASCUS, 11 as positive for malignancy squamous cell carcinoma (SCC).

9 NIL cases turned out to be chronic cervicitis, of the 18 LSIL cases 14 were reported as CIN I,2 as CIN II,1 as CIN III,I as chronic cervicitis.

1 ASCUS turned out to be CIN I,3 HSIL turned out to be 1 CIN II & 2 CIN III respectively.All the 11 cases of positive for malignancy turned out to be SCC in biopsy. In our study 90% of NIL ,77% of LSIL,100% POSITIVE FOR MALIGNANCY,100% of HSIL correlated with biopsy, reports were more concordant for NIL,High grade lesions &SCC.

Correlation of grading cervical lesion with morphologic criteria in cervical biopsy was statistically significant with p value 0f 0.001.

HPV DNA TYPING FOR 16 AND 18

In HPV DNA typing of 41 cases, 20 cases showed positivity for HPV DNA,Of which 18 were HPV DNA 16 positive and 2 were HPV DNA 18 positive.HPV DNA 16 was positive in 43.9% of cases, while HPV DNA 18 was positive in 4.9% of cases.

In relation to cytology, HPV DNA was positive in 1(11.1%)of the 9 NIL,6 (38.9%) Of the LSIL, all the 3(100%)HSIL,10 (90.0%)of SCC,none in ASCUS.

Of the total 20 HPV DNA positive cases,1 was cervicitis,5 were CIN I,2 were CIN II ,2 were CIN III and 10 were SCC.

And thus HPV DNA were positive in 90.9% of SCC,11.1% of cervicitis, 33.3% of CIN I cases,80% of CIN II&CINIII cases.

In our study, the correlation of HPV DNA positivity with morphological criteria of cervical biopsy was statistically significant with p value 0f <0.001.

HPV E6 mRNA EXPRESSION

Of the total of 41 cases,20 turned out to be HPV DNA Positive ,of which only 18 cases showed HPV mRNA expression.Comparing with cytology, HPV E6 mRNA was positive in 1(11.1%) of 8 NIL cases,5(29.4%) of 18 LSIL,2(66.6%) of 3 HSIL,10(90.9%) of 11 SCC.Correlation was statistically significant with p value <0.001.

Comparing with HPE,HPV E6 mRNA positive in 1(10.0%) of 10 chronic cervicitis,10(90.9%) of 11 SCC,5(33.3%) of 15 CINI,2(40%) of 4CIN II&CIN III cases.

Correlation of HPV E6 mRNA expression with morphological criteria of cervical biopsy results were statistically significant with p value<0.001.

In our study, there were 10 cases of chronic cervicitis, cytology correlated with 9 cases. But HPV DNA and E6 mRNA were found positive in one case of chronic cervicitis reported in biopsy. Senstivity of cytology was about 90% and specificity was 97% The one case which was positive for HPV &E6mRNA can be due to the latent infection of HPV which cannot be picked up in cytology /HPE that is the stage before which the HPV infection could produce morphological changes. Also if the biopsy was not representative site, it can lead to such result..E6 expression in that one case gives us a clue that lesion is progressive and this particular patient should be followed up regularly.Sveinung WergelandSørbye et al[31] in his study he emphasizes that negative cervical biopsy require follow-up before resumption of routine screening. HPV mRNA testing was as sensitive but more specific than cytology and it also had higher positive predictive value. Follow up of negative cervical biopsy in his study some cases developed cancer, it may be the first biopsy or histological slide may not have been representative for the underlying disease or a micro lesion difficult to detect by colposcopy.

COMPARISON OF EFFICACY OF CYTOLOGY, HPV DNA, HPV E6 mRNA IN LOW GRADE LESION (CINI)(TABLE 8)

In our study, there were 15 cases of CINI, among which cytology was able to pick up 14 cases, but the HPV DNA showed positivity in only 5 of these cases, out of these 5cases of HPV DNA positivity, HPV E6 mRNA was also expressed in these 5 cases. Progression of lesion among this 15 CIN I to high grade was indicated by HPV E6 mRNA positivity, that is only 5 of the CIN I is going to progress. And these cases need to be followed up frequently.

So in our cytology and HPV DNA also appears to be more sensitive in detecting lesions than HPV E6 mRNA but it lacks specificity. Thus specificity and the positive predictive value of HPV E6 mRNA(100%) was higher than cytology.

Thus in our study of low grade lesions the sensitivity and specificity of cytology was 93.3% and 92.3%,for HPV DNA 33.3% and 96.15%,for HPV E6 mRNA it was 33.3% and 100% respectively.

Marc Arbyn et al[23] in his study of triage of women with low grade cervical lesions have concluded that HPV mRNA testing was more sensitive and specific than repeat cytology in triage of women with LSIL cytology.In addition, the HPV mRNA test showed higher PPV. These data indicate that the HPV mRNA test is a better triage test for women with LSIL than repeat cytology.

COMPARISON OF EFFICACY OF CYTOLOGY ,HPV DNA,HPV E6 mRNA IN CASE OF HIGH GRADE LESIONS(CIN II&CINIII)(TABLE 9)

In our study there were 5 high grade lesions(CIN II&CIN III),cytology showed positivity in only 3 cases whereas HPV DNA was positive in 4

and HPV E6 mRNA was positive in only 2 of the lesions Thus the cytology was less sensitive than HPV DNA.E6 mRNA was also less sensitive than HPV DNA as it was positive in only 2 cases . This result is expected since not all HPV infection is going to express E6 mRNA.And we know that about 30-40% of high grade lesions also going to regress.

The presence of HR HPV is necessary, but not sufficient to cause cervical lesions (Bertuccio et al., 2011)[24]HPV DNA was more sensitive in detecting high grade lesions than the cytology.

Rate of detection for HPV DNA was 22.2% and for HPV E6 mRNA 40% in high grade lesion.Thus in our study sensitivity and specificity of cytology,HPV DNA and E6 mRNA was 60 and 94.4%,80 and 97.2%,40% and 100% respectively.

COMPARISON OF EFFICACY OF CYTOLOGY,HPV DNA,HPV E6 mRNA IN INVASIVE CANCERS(TABLE10)

In our study there were 11 cases of biopsy proved SCC,cytology showed 100% sensitivity in detecting SCC ,all 11 cases were reported as positive for malignancy in cytology. But only 10 cases were detected by HPV DNA and E6 mRNA, one case was negative. This may be due to sampling error, necrotic tissue sent for test, causes other than HPV or may be due to serotypes other than 16and 18 which was not used in the test.

Thus the sensitivity and specificity for cytology,HPV DNA and HPV E6 mRNA for SCC in our study was 100% and 100%,90.9%and 100%,90.9% and 100% respectively.

Table 11:Comparing The Sensitivity And Specificity In Our Study With Other Studies In Low Grade Lesions

		Mariobenevo et al(83)	Sveinung wargeland etal(84)	Miriam reuschenbach et al(85)	Our study-41 cases
Cytology	Sensitivity	92%	86%	86%	93.3%
	specificity	21%	80%	60.4%	92.3%
HPV DNA	sensitivity	85%	32.7%	91.5%	33.3%
	specificity	13%	84%	56.4%	96.15%
HPV E6 mRNA	Sensitivity	62%	66.3%	88.4%	33.3%
	Specificity	75%	90%	71.2%	100%

Table 12:Comparison Of Sensitivity And Specificity In Our Study With Other Studies In Case Of High Grade Lesions

		Mario benelov et al(83)	Sveinung wargeland et al(84)	Miriam reushenbach et al(85)	Our study-41 cases
cytology	sensitivity	53%	92%	80%	60%
	specificity	91%	86%	74.8%	94.4%
HPV DNA	sensitivity	96%	73%	96.4%	80%
	specificity	30%	70.8%	49.1%	97.2%
E6 mRNA	sensitivity	68%	63%	95.5%	40%
	specificity	95%	89.5%	63.4%	100%

Thus our study significantly correlated with other studies in low grade as well as high grade lesions.

Overall view, in our study the cytology based screening was reliably sensitive in detecting all lesions, expect for some lack of specificity in high grade lesions, this can be attributed by the fact that ours is a tertiary care with well trained technicians for pap smear staining and well experienced pathologist for pap reporting. We all know that pap smear screening effort will be effective when implemented in the reliable infrastructure.[28]

The result from DNA and RNA based assay was found to be associated well with the grades of lesion.

In study by Molden et al[29] E6 and E7 mRNA transcripts are usually expressed at low levels in the differentiated exfoliating epithelial cells, whereas a dedifferentiated neoplasia would express more E6/E7 in the surface epithelium. The present study found that E6/E7 mRNA expression correlated with the severity of cervical lesion Pierry et al.[30] found that 6% of benign cases, 22% of Grade 1cervical intraepithelial neoplasia, 83% of Grade 2 cervical intraepithelial neoplasia and 93% Grade 3 cervical intraepithelial neoplasia had positive E6/E7 mRNA in women under 30 years of age . In our study,33.3% of CIN I,40% Of CIN II&III,90.9% Of SCC had positive E6mRNA .

In this study, HPV E6 mRNA was negative in 3 histologically confirmed high grade CIN. It is assumed that some Grade 2 cervical intraepithelial neoplasia cases may clear their infections or cell abnormalities spontaneously restraining E6/E7 mRNA expression (Ronco et al., 2006, 2007[18,19]; Confortini et al., 2010[17]), but they require time to recover completely. It is therefore possible that Grade 2 cervical intraepithelial neoplasia cases with negative E6/E7 transcripts could be more likely to regress or less likely to progress toward cancer, as HPV infection could have been cleared or viral gene expression is very low. Regression of Grade 2 cervical intraepithelial neoplasia is frequent among younger women referred to colposcopy (Ronco et al., 2008[20]).

In some lesions DNA from HPV was detected more frequently than the E6 mRNA transcripts. The results probably reflected the transient nature of HPV infection, only a few of which produce precancerous lesion. We are also not able to exclude the possibility in these patients with positive DNA and negative E6 mRNA will never progress to invasive cancer. But certainly the timing of follow up can be less.

CONCLUSION:

In high grade lesion and malignancy, proportion of detection of E6 transcripts from HPV types 16 & 18 where almost same degree as DNA detected . But in lowgrade lesion with HPV DNA positivity E6 mRNA detection was significantly low with increasing severity of lesion expression of E6 mRNA was found to increase . low positivity rate and high specificity make E6 mRNA better than HPV DNA in a triage setting . Hence E6 mRNA assay can be useful as a biomarkers for potentially persistent High risk HPV infections that requires intervention and treatment.

LIMITATIONS OF THE STUDY:

The population of RNA study done was in slightly different from the screening population as those with abnormal cytology and colposcopy were chosen.Also study population was relatively small to compare the efficacy of HPV DNA and E6mRNA. Larger clinical trials are required to confirm these values.

ABBREVIATIONS

WHO: World health organization
HPV- Human papilloma virus
DNA- Deoxy ribonucleic acid
mRNA- messenger Ribonucleic acid
ASCCP- American society for colposcopy and clinical pathology
HPE- Histopathological examination
VIA- visual inspection with acetic acid
VILI- visual inspection with lugol's iodine
LGL- Low grade lesion
HGL- High grade lesion
ASCUS- Atypical squamous cells of unknown significance
LSIL- low grade squamous intraepithelial neoplasia
HSIL- high grade squamous intraepithelial neoplasia
CIN- cervical intraepithelial neoplasia
SCC- Squamous cell carcinoma
PCR- Polymerised chain reaction

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