RESEARCH ARTICLE

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Prevalence of Transcriptionally Active *HPV16* and *HPV18* infection in Anogenital Warts: A Study in Sikkim, India

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Abstract

Background: Human papillomavirus (HPV) is the most prevalent sexually transmitted infection globally and is causally related to anogenital and oral cancers, with 311,000 associated deaths recorded in men and women every year. The presence of oncogenic HPV genotypes in anogenital warts is reported by few studies, while many groups have reported an associated higher risk of anogenital and other cancers in patients with anogenital warts. This study aimed to detect *HPV16* and *HPV18* DNA and mRNA markers in anogenital warts to assess infection and viral persistence. **Method**: A cross-sectional study design was adopted to enroll 50 consenting men and women presenting with anogenital warts at the dermatology clinics of two existing referral hospitals in Sikkim. Samples were processed for DNA and RNA extraction, cDNA conversion, followed by qPCR-based amplification for *HPV16* and *HPV18* E6/E7 DNA and mRNA. **Result:** High presence of *E6/E7* genes of *HPV16* and *HPV18* with DNA and mRNA was observed in the exfoliated anogenital warts tissue samples, evaluated using quantitative PCR (qPCR). *HPV16E6* and *HPV18*E6 DNA was present in 75.5% (37/49) and 26.6% (13/49) of the samples, while *HPV16E6* and *HPV18E6* mRNA was present in 62.0% (31/50) and 52.0% (26/50) of the samples, respectively, 18.0% (9/50) of the samples tested positive for *HPV16E7* mRNA, while none were positive for *HPV18E7* mRNA. **Conclusion:** High prevalence of *HPV16* and *HPV18* seen in males and females with Anogenital warts in the present study re-emphasizes the significance of including men in HPV screening and vaccination programs, and importance of testing oncogenic HPV genotypes in anogenital warts to improve the overall reduction of clinical manifestations associated with HPV.

Keywords: Anogenital warts- *HPV16- HPV18- E6/E7* mRNA

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Introduction

Anogenital warts (AGWs) are lesions of the anogenital region caused by highly infectious Human papillomavirus (HPV) [1]. It can present at one or more of the anogenital sites like vagina, anus, perineum, perianal area, cervix, vulva, penis, scrotum or urethra, as single or multiple papules [1, 2]. AGWs have a very high transmission rate with an estimated 10% lifetime risk of occurrence in sexually active individuals [2]. Global incidence of AGWs occurs in a range of 160 to 289 (new and recurrent) cases per 100,000 individuals (men and women), and prevalence with a median 195 cases per 100,000 individuals [3]. Using a two week daily log of 200 clinicians practicing in six different regional centers in India, the estimated prevalence of AGWs was found to be 1.07 % with 95% CI: 0.97–1.17 [4]. Anogenital warts are more prevalent in promiscuous individuals and are highly infectious with 65% of the sexual partners of individuals with AGWs developing the condition within 3 weeks to 8 months [5].

AGWs patients have a comparatively higher risk of developing anogenital, and other HPV associated cancers [6-9]. A study on 50,000 Danish men and women with genital warts reported an increased lifetime risk of developing anogenital cancers with Standard Incidence Rate [SIR] for anal cancer in men as 21.5 and female as 7.8, vulvar-14.8, vaginal-5.9, cervical-1.5, penile-8.2 and Head and Neck cancers (HNCs), 2.8 [6]. Another study on Swedish Inpatient Register data collected between 1965 and 1999, and a median follow up period of 13 years, reported SIR for vulva-10.2, vagina-12 and penis-21.9 respectively [7], evidently supporting the cancer risk associated with AGWs.

The low risk genotypes, HPV6 and HPV11 are casually related with AGW, however, high risk oncogenic HPV genotypes are also known to be present [10-14]. HPV genotypes other than HPV6 and HPV11, reported in AGWs include HPV type 16,18,33,52,55 and 58, with

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the highest presence reported for *HPV16* [11-16], the genotype that causes 50% of all HPV related cancers. It is hypothesized that patients with AGWs progressing into malignancies might be harboring the oncogenic high risk genotypes of HPV, however, limited studies have evaluated the presence of high risk genotypes in AGWs. In our knowledge, this is the first study from India on molecular profiling of AGWs for oncogenic HPV genotypes.

The presence of HPV E6/E7 mRNA reflects active viral infection and is thus considered a gold standard indicator for cancer transformation risk, higher expression of E6 and E7 oncoproteins is often reported in HPV related cancers [21-22]. In the present study, infection by oncogenic *HPV16* and *HPV18* genotypes was evaluated with quantitative PCR targeting the E6/E7 DNA and mRNA.

Materials and Methods

Study design and patient recruitment

A cross sectional study design was adapted for enrolling consenting male and female participants with anogenital warts, from outpatient Dermatology Department of the two Referral Hospitals, namely, Central Referral Hospital (CRH) and Sir Thutob Namgyal Memorial Hospital (STNM) in the State of Sikkim, India. The study was approved by the Institutional Ethics and Review committee of Sikkim Manipal University with reference number, SMIMS/IEC/C/2020-37. Men and women with AGWs of age 18 years and above were recruited with coordination of the counsellors at the Integrated Counselling and Testing Centre (ICTC) for sexually transmitted diseases of both the Hospitals. Sociodemographic data on general health, dietary habits and substance behavior, with awareness on sexually transmitted diseases were collected in approved proforma. Superficial cell scrapings from the warts tissue was collected and transported in 1X Phosphate Buffered Saline by maintaining cold chain to the Biochemistry Department of Sikkim Manipal Institute of Medical Sciences and stored at -20ºC until further processing for all molecular analysis.

Nucleic acid extraction and cDNA conversion

The exfoliated cells were processed for nucleic acid extraction using trizol method for RNA as described by Rio et al. [24] and phenol chloroform method for DNA as described by Sontakke et al. [25]. The DNA and RNA obtained were quantitated using nanodrop Spectrophotometer (Eppendorf) and subsequently, depending on the yield a minimum of 200ng and maximum of 500ng of RNA from each sample was converted to cDNA using High-Capacity reverse transcription kit (Applied Biosystems-Cat No 4368814) in a final reaction volume of 20µl. The cDNA conversion was done using an initial heating at 25ºC for 10mins followed by incubation at 37ºC for 120mins, then 85ºC for 5 mins and final hold at 4ºC and the cDNA so obtained was stored at a final concentration of 10ng per µl.

Primers and qPCR

The primers for quantitative PCR (qPCR) amplification were designed in house using primer3Plus software (Box 1). The quality of the primers were checked for single peak amplification using melt curve analysis to rule out any nonspecific amplification and thereby selected for the study. qPCR was done on CFX96 Bio-Rad real-time PCR detection system using 5ul of 2XTB green pre-mix (Takara, Cat no RR420L), 100mM primers and 5ng of template in a final volume of 10ul. The reaction cycle comprised of initial denaturation at 95ºC for 10 mins followed by 40 cycles of reaction steps at 95ºC for 15 secs, 60ºC for 15 secs and 72ºC for 15 secs, melt curve generated using temperature from 60ºC to 95ºC, increased in an increment of 2º C after every 5 seconds. Positive, negative and no template control (NTC) were included in each run, and *GAPDH*, 5sRNA were used as internal controls (ICs). Two reactions were set for each sample, and the average of corresponding Ct values was used in further analysis and reporting. Serial dilutions of HPV positive cell line (Hela and SiHa) were amplified and the Ct corresponding to the lowest dilution showing amplification was used as Ct cut offs for positive amplification.

Statistical Analysis

All patient data was recorded in Microsoft Excel. Statistical analysis was carried out using Statistical Package for Social Sciences, SPSS version 25.0' (IBM Corp, Armonk, NY,USA). The frequency counts and percentages for various sociodemographic parameters

Box 1. Primer Details

Gene Name	Sequence	Amplicon size (bp)	Reference sequence
HPV16-E6	F: CTGTCAAAAGCCACTGTGTCC	100	NC_001526.4
	R: AGACATACATCGACCGGTCC		
HPV16-E7	F: TGCAAGTGTGACTCTACGCT	111	NC_001526.4
	R: AGAACAGATGGGGCACACAA		
HPV18-E6	F: CAGACTCTGTGTATGGAGACA	101	GQ180792.1
	R: TGCTGGATTCAACGGTTTCTG		
HPV18-E7	F: GCCCCAAAATGAAATTCCGGT	113	GQ180792.1
	R: TCGTCGGGCTGGTAAATGTT		
GAPDH	F: ACAGCCTCAAGATCATCAGCA	118	NM_001357943.2
	R: ATGGCATGGACTGTGGTCAT		

was calculated and tabulated and further correlated and analyzed. Chi Square test was used to define the association between HPV positivity and Sociodemographic features. The association of age of first sexual exposure, alcohol behavior, smoking and gender with odds of having HPV infection was analyzed using binary logistic regression with bootstrapping to compensate for the small sample size.

Results

General characteristics of the study population

Anogenital warts (AGWs) occur in sexually active young individuals, with maximum presentation (~80%) seen between 17 to 33 years of age [3]. The mean age of the study participants was 31 (± 10.2) years, 80.0% were below 40 years of age, no specific gender inclination was noted in this study with 26 men and 24 females participants (Table 1). Participants were mostly from the east district of Sikkim where both the referral Hospitals of Sikkim are located. All study participants had their first sexual exposure ≤ 30 years of age, wherein most were unmarried, sexually active. Only 4 participants have self-reported having more than one lifetime sexual partner.

Alcohol consumption was reported by 80% of the participants with median age of initiation at 20 years (±3 years), while 40.0% reported smoking with median age of initiation at 19 years (±5.4 years), all smokers also reported alcohol consumption.

Most of the participants were employed 76.0% (38/50), and only 24.0% (12/50) were unemployed. As per Kuppuswamy Scale 2019, majority of the study participants were from middle class 40.0% (20/50) followed by lower middle class 28.0% (14/50), and 18.0% (9/50) were from upper lower class, 12.0% (6/50) were from upper class and 2.0% (1/50) belonged to lower class (Table 1).

The genital warts were in penis, perineum, vagina, and anus, with a distribution of 22 (44.0%), 13 (26.0%), 8 (16.0%) and 7(14.0%) respectively. Three individuals presented with AGW with a standing time of over one year, of which two were recurrent cases, two others self-reported history of AGW diagnosis and treatment in their partners. There was one married couple with both partners having anogenital warts: the husband had penile wart standing from over 90 days and his wife had multiple vaginal warts that developed in the past 45 days.

Presence of HPV 16 and HPV18 E6 DNA

Molecular testing for HPV DNA complements the traditional microscopic methods and is widely used to test the direct physical presence of HPV. DNA based amplification of *HPV16* and *HPV18 E6* gene was done for 49 samples, one sample could not be amplified due to poor DNA yield. High presence of *HPV16* was noted in the study participants with 75.5% (37/49) showing amplification for *HPV16*E6 DNA, while 26.6% (13/49) were positive for *HPV18* HPV DNA, and a combined prevalence of 83.7% (41/49) for both genotypes. The age group between 18-25 years showed the highest positivity for *HPV16E6*-DNA, 83.3% (15/18) (Table 2). Further,

Table 1. Sociodemographic Features of Participants

Characteristics	Frequency (%)
Age group (years)	
18-25	18 (36)
26-35	15 (30)
36-45	12 (24)
>45	5 (10)
Gender	
Male	26 (52)
Female	24 (48)
Employment	
Employed	38 (76)
Unemployed	12 (24)
Marital Status	
Married	26 (52)
Unmarried	22 (44)
Divorced	1 (2)
Widowed	1 (2)
Socioeconomic Status (as per Kuppuswamy scale 2019)**	
Upper Class	6 (12)
Middle Class	20 (40)
Lower Middle class	14 (28)
Upper Lower Class	9 (18)
Lower Class	1 (2)
Religion	
Hinduism	35 (70)
Buddhism	14 (28)
Islam	1 (2)
Body Mass Index (BMI)	
Obese	6 (12)
Overweight	9 (18)
Normal	33 (66)
Underweight	2 (4)
Diet	
Vegetarian	2 (4)
Non-vegetarian	48 (96)
Alcohol consumption	
Yes	40 (80)
No	10 (20)
Smoking	
Yes	20 (40)
No	30 (60)
Number of sexual partners	
Single	46 (92)
Multiple	4 (8)
Aware of Sexually Transmitted Diseases (STD)	
Yes	25 (50)
No	25 (50)

**The socioeconomic status was determined using Kuppuswamy reference scale revised in 2019

Table 2. *HPV16* and *HPV18* Positivity with DNA and mRNA Markers

HPV16/18 E6 DNA based type specific prevalence (n=49*)				
	HPV16 DNA		*HPV18*DNA	
Anogenital location of wart	Positive 37 (75.5%)	Negative 12 (24.5%)	Positive 13 (26.6%)	Negative 36 (73.5%)
Anal (6)	5 (10.2%)	1 (2.0%)	2 (4.1%)	4 (8.2%)
Penile (22)	17 (34.7%)	5 (10.2%)	6 (12.2%)	16 (32.7%)
Perianal (13)	11 (22.4%)	2 (4.1%)	3 (6.1%)	10 (20.4%)
Vaginal (8)	4 (8.2%)	4 (8.2%)	2 (4.1%)	6 (12.2%)
*HPV16/18 E6*mRNA based type specific prevalence (n=50)				
	HPV16 E6 mRNA		*HPV18 E6* mRNA	
	Positive 31 (62.0%)	Negative 19 (38.0%)	Positive 26 (52.0%)	Negative 24 (64.0%)
Anal (7)	5 (10.0%)	2 (4.0%)	5 (10.0%)	2 (4.0%)
Penile (22)	15 (30.0%)	7 (14.0%)	11 (22.0%)	11 (22.0%)
Perianal (13)	8 (16.0%)	5 (10.0%)	6 (12.0 %)	7 (14.0%)
Vaginal (8)	3 (6.0%)	5 (10.0%)	4 (8.0%)	4 (8.0%)
*HPV16/18 E7*mRNA based type specific prevalence (n=50) #				
	HPV16 E7 mRNA			
	Positive 9 (18.0%)		Negative 41 (82.0%)	
Anal (7)	0 (0.0%)		7 (14.0%)	
Penile (22)	5 (10.0%)		17 (34.0%)	
Perianal (13)	1 (2.0%)		12 (24.0%)	
Vaginal (8)	3 (6.0%)		5 (10.0%)	
Age wise distribution of participants and respective positivity (%) with HPV16 DNA , HPV18 E6/7 DNA and mRNA markers				
Age group (years)	Positivity *HPV16*-DNA	Positivity *HPV16*-E6mRNA	Positivity *HPV18*-DNA	Positivity *HPV18-E6*mRNA
18-25 (n=18)	15 (83.3 %)	8 (44.4%)	6 (33.3%)	10 (55.6%)
26-35*(n=16)	10 (62.5)	12 (75%)	3 (18.8 %)	8 (50.0.3%)
>36 (n=16)	12 (75.0 %)	11(68.8%)	4 (25.0%)	8 (50%)
Socioeconomic group wise distribution of participants and respective positivity (%, within the group) with HPV16 DNA, *HPV18 E6/7* DNA and mRNA markers				
Age group (years)	Positivity HPV16-DNA	Positivity HPV16-E6mRNA	Positivity HPV18-DNA	Positivity HPV18-E6mRNA
Upper Class (n=6)	3 (50.0%)	5 (83.3%)	0 (0.0%)	4 (66.7%)
Middle and lower class (n=44)	34 (77.3%)	26 (59.1%)	13 (29.5%)	22 (50.0%)
Gender Wise Per Cent positivity with HPV16 and HPV18 DNA and mRNA markers				
Gender	*HPV16E6/E7* DNA (37/49)	*HPV16E6-* mRNA (31/50)	*HPV18 E6/E7* DNA (13/49)	*HPV18E6-* mRNA (26/50)
Men	19 (51.3%)	18 (58.1%)	8 (61.5%)	14 (53.8%)
Women	18 (48.6%)	13 (41.9)%	5 (38.5%)	12 (46.2%)

*one sample dropped due to low DNA yield; #None of the samples tested positive for HPV18E7 mRNA

perianal warts with [84.6% (11/13)] followed by penile warts [77.3% (17/22)] showed highest positivity with *HPV16E6*-DNA, no such location specific prevalence for *HPV18 E6*-DNA was noted (Table 2).

Presence of HPV16 and HPV18, E6/E7 mRNA

RNA based HPV markers are presented differently with latent and active form of infection, and expression of E6/E7 mRNA transcript is often reported to increase manifold with persistence of infection. Anogenital wart samples analyzed in the present study were found to align to this hypothesis and showed difference in positivity between E6 and E7 mRNA markers for both genotypes evaluated. *HPV16-E6* mRNA was positive in 62.0% (31/50), while 18.0% (9/50) were positive for *HPV16-E7*mRNA transcript (Table 2). *HPV18-E6*mRNA was present in 52.0% (26/50) samples, none of the samples showed positive amplification for *HPV18-E7*mRNA (Table 2). The positivity with E6 mRNA of both *HPV16* & 18 genotypes was highest in penile warts, 68.2% (15/22) and 50.0% (11/22) respectively, while the positivity with *HPV16E7*mRNA within penile AGWs was 55.6% (5/9),

Table 3. Binary Logistic Regression Analysis for Sociodemographic Features and HPV Positivity

	HPV16 DNA		*HPV16*mRNA		*HPV18* DNA		*HPV18* mRNA	
	Odds Ratio (95% CI)	Sig (P value)	Odds Ratio (95% CI)	Sig (P value)	Odds Ratio (95% CI)	Sig (P value)	Odds Ratio (95% CI)	Sig (P value)
Age of sexual debut	0.980 (0.916-1.050)	0.57	1.221 (0.993-.501)	0.06	1.005 (0.879-0.148)	0.95	1.079 (0.942-1.235)	0.27
Alcohol	1.182 (0.227-6.149)	0.84	3.310(0.619-7.697)	0.16	1.355 (0.262-7.016)	0.72	0.462 (0.098-2.184)	0.33
Smoking	.158 (0.020-1.258)	0.08	0.160 (0.024-1.068)	0.06	.922 (0.163-5.219)	0.93	7.675 (1.252-7.062)	0.03
Gender	2.275 (0.399-12.964)	0.36	1.342(0.262-6.874)	0.72	0.538(0.104-2.784)	0.46	0.345 (0.066-1.803)	0.21
Age	0.980 (0.916-1.050)	0.57	1.022 (0.954-1.094)	0.53	0.985 (0.912-1.063)	0.70	0.946 (0.879- 1.018	0.14
Nagelkerke R Square	0.15		0.28		0.03		0.19	
Cox & Snell R Square	0.10		0.20		0.02		0.14	
Chi square goodness of fit-Omnibus model (sig)	5.273 (p= 0.383)		11.359 (p=0.045)		1.121 (0. 0.952)		7.454 (0.189)	

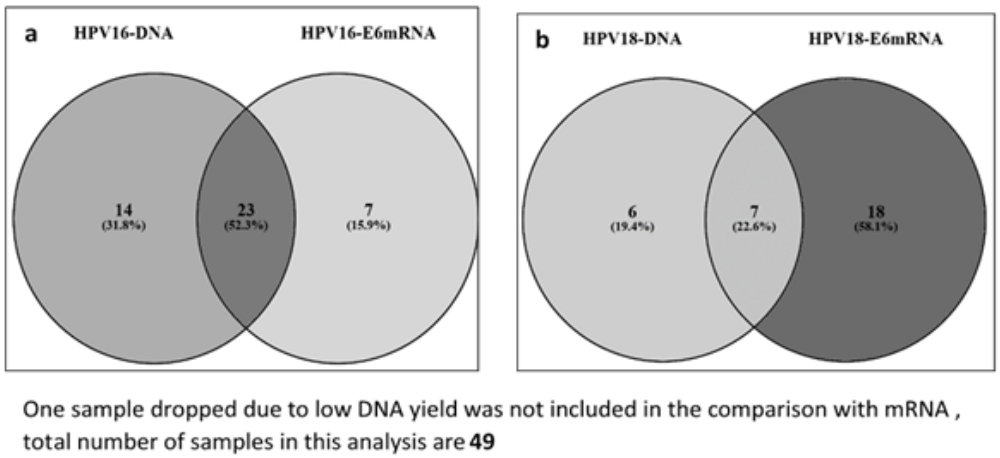

Figure 1. Comparative Presence of DNA and mRNA of *HPV16* and *HPV18* Genotypes

33.3% (3/9) within vaginal and 11.1% (1/9) in Perianal AGWs.

Positive infection by DNA Vs mRNA

We noted a significant difference in positivity with HPV DNA and mRNA markers for the two HPV genotypes evaluated in this study (Figure 1). Among the study participants, 75.5% (37/49) were positive for E6-DNA and 61.2% (30/49) were positive for *HPV16-E6* mRNA, and 89.8% (44/49) were positive for either DNA, or mRNA or both the markers [Figure 1a]. Similar difference in *HPV18 E6* DNA and mRNA positivity was noted, comparatively higher number of samples 51.0% (25/49) were found to be positive with *HPV18E6*-mRNA, while with *HPV18 E6*-DNA 26.5% (13/49) positivity was noted, an overall positivity of 63.3% (31/49) with either DNA, or mRNA or both the markers [Figure 1b].

Sociodemographic parameters and HPV positivity

Smoking and alcohol consumption are known risk factors for developing AGWs. Among the participants, 80.0% (40/50) self-reported the habit of alcohol consumption, of which, in 82.5% (33/40) individuals, *HPV16 E6* DNA or mRNA was present and in 77.5% (31/40) *HPV18 E6* DNA or mRNA was present. Alcohol usage was found to be significantly associated with the presence of *HPV16 E6*mRNA using chi square test of independence, X^2 df=1, X^2 = 5.5, (p=0.01, at 95% CI), no stastical significant association was seen for *HPV16-E6*DNA.

Smoking was self-reported by 40.0% (20/50) of the participants, all of them were positive for *HPV16* with either E6 DNA or mRNA and 70.0% (14/20) were positive for *HPV18 E6* DNA or mRNA. Binary Logistic regression analysis with bootstrapping (1000 samples) was done to model the predictive factors for HPV positivity. The model was used to test for age of presentation with AGW, age of sexual debut, alcohol behavior, smoking and gender as risk factors for infection by *HPV16* &18 E6/E7 DNA and mRNA. The results with model parameters are listed in Table 3. Alcohol had a high odds ratio of 3.310 (p=0.162) for infection with *HPV16 E6*mRNA and smoking for infection with *HPV18 E6* mRNA with an odds ratio of 7.675 (p=0.028). Age of first sexual exposure was found to be an important risk factor for infection with *HPV16E6*mRNA with an odds ratio of 1.221, p=.058.

Socioeconomic status was found to be an important determinant in occurrence of AGWs with 88.0% (44/50)

of participants coming from middle and lower class, of which 34/44 (77.3%) were positive for *HPV16-E6*DNA (Table 2). There is a global consensus on poor living standards with compromised awareness and practice of hygiene, as important determinant in increasing the risk of HPV infection.

The maximum positivity with *HPV16-E6*DNA [15/18 (83.3 %)] and *HPV18-E6*DNA [6/18 (33.3%)] and *HPV18-E6*mRNA [10/18(55.5%)] was seen in individuals aged between 18-25 years, while *HPV16-E6*mRNA [12/15) 80%] was highest in the age between 26 to 35 years (Table 2).

Discussion

Genital warts are common in young sexually active individuals, are highly transmitted between sexual partners and are associated with risk for malignant transformation. The casual association of HPV6 and HPV11 with AGWs is well known, however, the presence of high risk genotypes is less evaluated. In the present study, high presence of *HPV16* and *HPV18* genotypes in AGW was seen with both DNA and mRNA markers, studies from other countries, although limited in number, have reported similar findings [10-16]. In our knowledge, this is the first study from India on molecular profiling of AGWs for oncogenic HPV genotypes, earlier a study by Singh et al. [17], analyzed 22 AGW formalin fixed tissue samples for presence of HPV6 and HPV11, in this study 63.6% samples tested positive for HPV DNA , high risk genotypes were not evaluated [17].

The highest presentation was seen for Penile warts 44.0% (22/50), it also corresponded with maximum positivity with DNA and RNA markers for both the genotypes evaluated, however, further studies are needed to establish the global trend in precise location specific genotypic prevalence of HPV in AGWs.

AGWs occur in younger individuals, usually during years with peak in sexual activity, similar age wise presentation was noted in the present study with 84.0% of the AGW patients below 40 years of age. A higher prevalence of AGWs in males compared to females is reported by other studies [4-5], however, in the present study participant showed no such gender specific inclination.

Molecular testing of HPV using DNA and mRNA markers may give different results, an outcome believed to be in part related to the strict regulation of replication and transcription of viral genes depending on nature of viral interaction with the cellular microenvironment [18]. Difference seen in the DNA Vs RNA markers of viral gene, is reported to depend on factors like sample type, sampling technique, duration and persistence of infection [14-18]. A UK based study on 23 AGWs patients, reported a significant difference in HPV positivity and genotypic distribution with DNA and mRNA makers. Apparently, sampling was found to be a predominant factor behind these differences, the same patient tested differently for HPV genotypes depending on the sample; swab, whole wart tissue or micro-laser-dissected upper layer or lower tissue layers [14]. We have used superficially exfoliated cells directly from the wart tissue, the high positivity type with mRNA reflects the presence of actively transcribing virus in the sample. High presence of active infection by the oncogenic HPV genotypes in AGW samples seen in the present study, needs further evaluation to define its future clinicopathological implications.

Unequal presence of HPV DNA and mRNA was seen for the samples evaluated; this difference was more prominent for *HPV18* as compared to *HPV16*. The viral E6 and E7 oncogenes of high risk genotypes are known to act as oncogenes that disrupt cell cycle regulation and push the cells towards neoplastic proliferation [21]. Differential splicing of the bicistronicE6/E7 pre mRNA transcript is responsible for expression of E6I*, E6II* and E7 transcripts, less is known about the molecular interplay behind the selection of available splicing alternatives. In a study on cervical samples, *HPV16 E7* mRNA expression was reported to be correlated to progression to HSIL/CIN2+, 87.5% of samples positive for *HPV16* E7 mRNA progressing to HSIL/CIN2+, 60% of the samples were negative for *HPV16 E7* mRNA of which just one sample progressed to HSIL/CIN2+ [23]. Variation in expression of E6 and E7 transcript was noted in the present study, a comparatively lower number of samples tested positive for E7 transcripts as compared to E6, if this phenomenon is specifically associated with risk of cancer transformation of AGWs needs further evaluation. High positivity with E6 mRNA observed in the present study and the associated risk of cancer observed in patients with AGWs reported by others [6-9], evidently emphasize on the need for further studies on deciphering the molecular mechanisms underlying the associated oncogenic changes.

Despite the high risk of malignant transformation, AGWs have failed to draw considerable attention in HPV screening and vaccination programs. The focus of most vaccination and screening programs in many countries is only on women, although, prevalence of HPV infection and associated pathologies is comparable in men and women [27]. Apparently, males reportedly have higher oral HPV prevalence with 11.5% as compared to 3.2 % in females [26-28], notably, oral *HPV16* infection is reported to occur 6 times more often in males than females [26-28], also, a recent study published in Lancet claims that one every five men globally is infected high risk oncogenic HPV genotypes [29].

With a general increase in HPV associated cancers in men and overall rise in deaths associated with anal cancers in the past few decades [30], there is a need for studies to generate data focusing on this line from different regions across the globe.

High positivity and persistence of *HPV16* and *HPV18* anogenital warts signifies the need to conduct studies on screening of genital warts for oncogenic genotypes to establish the regional prevalence data for framing HPV vaccination and treatment policies.

Apparently, infection by HPV is often cleared autonomously, regression of even high-grade lesion is a common observation and thereby, long prospective studies are needed to clearly define the associated risk factors leading to development of cancer in AGW patients. Our study findings warrants, larger community based study

to evaluate scope of interventions to ensure equitable vaccination coverage of men and women to effectively control HPV related malignancies in both men and women.

Author Contribution Statement

All authors have made substantial contributions to the work and participated sufficiently in the intellectual content, conception and design of this work or the analysis and interpretation of the data (when applicable), as well as the writing of the manuscript, their names are listed in the manuscript.

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Any Conflict of Interest

The authors declare that there are no conflicts of interest

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