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MOLECULAR DIAGNOSTIC ASSAY OF CERVICAL CANCER FOR THE DETECTION OF HIGH RISK HUMAN PAPILLOMAVIRUS TYPES 16 AND 18 BY POLYMERASE CHAIN REACTION

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ABSTRACT
Cervical Cancer (CC) accounts as one of the major gynecological malignancy among women, Human Papilloma Virus (HPV) being a prime etiological factor for causing cervical cancer. Hence, it is necessary to detect the prevalence of HPV in this population. This study was conducted to detect the prevalence of HPV 16 and 18 in North Karnataka population by the latest molecular tool Polymerase Chain Method. Between 2010-2012 women within the age group between 29-86 years visiting the hospital with chief gynecological complaints were considered for the study. A total of one hundred and thirty four samples were collected and out of these one hundred and twenty two samples were subjected to a molecular technique polymerase chain reaction using consensus primer at E6 region for HPV types 16 and 18.One hundred and one samples were positive for HPV 16 (82.78%) and ninety two were HPV 18 (75.40 %) and eighty (65.57%) were with 16/18.Chi Square test was performed and the findings showed significant association with age and post coital bleeding, age and inter menstrual bleeding, age and menorrhagia, age and menopause and lastly age and post menopausal bleeding except with respect to age and white discharge and also age and HPV types where there was no significant association. Present study confirms the high prevalence of HPV 16 and 18 in North Karnataka population. Hence identification and diagnosis of HPV subtypes causing cervical cancer by PCR method is recommended.

KEY WORDS: Cervical Cancer, Human PapillomaVirus, Polymerase Chain Reaction, Screening.

INTRODUCTION

Cervical Cancer (CC) is the most common Gynecological malignancy among women[1]. WHO has reported that among all cancers CC occurs to be about 12 % among all the female cancers in the world[2]. India records 1, 32,000 new cases and 74,000 deaths each year[3]. Incidence rate is seen as 26.1% in Chennai and 21.5% in Bangalore in South India which stands as the highest for cervical cancer among females[4]. CC is a malignant neoplasm emanating from cells originating in the cervix uteri. The cervix is a narrow portion of the uterus which joins at the anterior portion of the vagina[5,6]. Most CC begins in the cell lining of the cervix. There are two main types of cervical cancer *i.e.*, Squamous cell carcinoma arising from the Squamous cells and Adenocarcinoma arising in the glandular epithelial cells among which 80-90% of CC are Squamous cell carcinoma[7]. Human Papilloma Virus (HPV) is the most common virus for sexually transmitted infection and it has been found that persistent infection with high risk types is an essential etiological factor for causing Cervical Cancer (CC) (8-10). HPV belongs to the family Papillomavirus and is found commonly worldwide. HPV is a non enveloped circular double stranded DNA virus with a size of 52-55nm having a genome of 7,900 base pairs and replicates at the basal cells of stratified Epithelium[11-13]. The genome of HPV is differentiated into early gene known as E1, E2, E4, E5, E6 and E7 and the late gene as L1and L2.E6 and E7 are the major oncoproteins in the formation of oncogenic tumors. E6 oncoprotein cause damage to P53 where as E7 oncoprotein binds to Rb gene and inactivate them [14,15]. High risk Human Papillomavirus types 16 and 18 have contributed to about 70% of cancer occurring worldwide[16]. Asymptomatic infection is observed in the initial stages for HPV infection but later stages they give rise to symptomatic lesions leading to cancer [17,18]. Thus the relationship of HPV and CC has given the importance for the development for the detection of different HPV types [19]. The Knowledge of HPV as the high risk factor has led to the development of HPV DNA detection in CC and also in the development of new strategies for screening and effective vaccination programmes [20-22]. This study helps in the Molecular detection of HR HPV types 16 and 18 on cervical punch biopsy samples. The method used for HPV detection was PCR by using consensus primer targeting E6 region of the gene.

MATERIALS & METHODS

The study was approved by the Institutional ethical review committee. Written informed consent was obtained from

all the patients in their local vernacular and their identity was coded as to maintain their confidentiality.

Inclusion Criteria: Patients with unhealthy cervix [23].

Exclusion Criteria: Pregnancy, Hysterectomy.

Sample Collection

The samples were collected by punch biopsy from the confirmed cervical cancer patients and transported in a 2ml centrifuge tube containing Ambion's RNA later® and stored at -80ºC for further testing. HeLa, CaSki, SiHa cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune, India and these cell lines were further cultured and their genomic DNA was extracted.

DNA extraction

Genomic DNA was isolated from cervical tissue samples using the DNeasy Blood and Tissue kit (Qiagen, Cat. No. 69504) according to the manufacturer's instructions. The isolated genomic DNA was quantified using Bio Photometer (Eppendorf, Germany). Agarose gel electrophoresis (0.8%) was run to check the isolated genomic DNA of cervical tissue samples for the presence of DNA bands.

PCR for HPV

The genomic DNA extracted from punch biopsy were subjected to amplification by PCR for HPV Consensus primers,HPV16 and HPV 18 with specific primers obtained for the study which were done earlier (24).The primer sequence for HPV consensus were.

Forward 5' CGGTCGGGACCGAAAACGG

Reverse 3' AGCATGCGGTATACTGTCTC

The reaction was carried out in a 10µl reaction volume containing 0.5 µl of genomic DNA ,0.5 µl of each primer, 1.0µl of dNTP, 0.3µl Taq DNA polymerases, 1.0 µl of 10X Taq Buffer, (NEW ENGLAND BIO LABS) and total volume was adjusted to 10 µl using Nuclease-free water. The amplification was carried out in a Master cycler gradient (Eppendorf, Germany) with an initial denaturation temperature at 95°C for 30sec, followed by 30 cycles at 95°C for 20sec (cycle denaturation), primer annealing temperature was set depending on the annealing temperature (T_m) of each primer for 40sec, 68°C for 50sec (primer extension) and a final extension at 68°C for 5 min. PCR products were confirmed for their respective amplicon size on gel electrophoresis with standard 100bp ladder. The PCR product (10µl) was amplified by electrophoresis on 0.8 % agarose gel stained with ethidium bromide and visualized in UV transilluminator and analyzed by Gel Documentation. A product size of 240 bp for HPV cons gives a positive result for the presence of HPV types.

Type specific PCR for HPV 16 and 18

Samples which were positive to PCR for Consensus sequence at the E6 open reading frame were further subjected to type specific PCR for HPV 16 and HPV 18 respectively. The sequence for HPV 16 forward primer 5' ATTAGTGAGTATAGACATTA and 3' GGCTTTT GAC AGTTAATACA and for HPV18 were 5'ACTATGG CGCGCTTTGAGGATCCA and 3' GTTTCTGGCA CCG CAGGCA. SiHa and CasKi were used as a positive control for HPV16 and HeLa for HPV 18. A bp of 110 for HPV 16 and 335 for HPV 18 were visualized on 0.8% agarose gel with a 100bp ladder.

Statistical Analysis

Data was statistically analysed using SPSS statistical software version 16.0.Chisquare test was performed to know the significance difference between different age groups and risk factors. The tests were considered significant if P-value is ≤0.05.

RESULTS

A total of 134 punch biopsy samples were collected and out of these 122 were positive for HPV virus *i.e*., 91.0% and 12 did not give any results due to debris. Patients age range was between 29-86 years (mean 50 years).The chief complaints presented by the patients were 52(42.62%) women were post menopausal, 24 (19.67%) complained of post coital bleeding, 25 (20.49%) with intermenstrual bleeding, 67 (54.91%) patients complained with vaginal discharge, 13 (10.65%) with Menorrhagia & 63 (51.63%) with Menopause. The age at first coitus ranged from 10-26 years (mean 16 years), 19 women had a history of tobacco chewing (15.57%), 42(34.42%) women had undergone tubectomy and 7(5.73%) with intrauterine contraceptive method and only 1(0.81%) women had received oral contraceptives. Parity ranged from 1-9 (mean 4). Their FIGO staging was 1 stage with 19 (15.57%),stage 2 with 36 (29.50%),stage 3with 63(51.63%) and stage 4 with 4 (3.27)%.The histopathological diagnosis showed that about 115 (94.26%) were of squamous cell carcinoma and 7(5.73%) were of adenosquamous cell carcinoma. HPV 16 were of 101(82.78%) and HPV 18 were 92(75.40%) and HPV 16 and 18 were found to be about 80 (65.57%).

DISCUSSION

The relationship of HPV and CC has given the importance for the development for the detection of highly sensitive technique for the detection of high risk HPV in clinical diagnosis[19-25]. Distribution of HPV is varied in different regions and it is important to understand these differences so as to make strategies for screening and vaccination [21]. The study conducted was a prospective hospital based study using PCR technique. This study was carried out in three major hospitals where women came from a diverse population with certain Gynaecological ailments and where the specialist recognized their ailments and categorized them with different stages of cervical cancer [17].

In our study PCR was used as a diagnostic tool to analyse the samples and the prevalence of HPV infection. Among the patients for consensus primers was 88.52% and out of these 82.78% were positive for HPV 16, 75.40% were HPV 18 and 65.57% with HPV16/18 types (fig 1). Our population is very unique consisting of consanguinity it seems that the prevalence rate is very high, which means our population is at the high risk end. When these samples were statistically analysed it was found that there was no association with age and HPV types. The results of high prevalence in our study was in concurrence by the study done by Pavani Sowjanya *et al*., and Neerja Bhatla *et al*., [3,26]. But the study done by Partha Basu states that HPV 18 was not statistically significant in their study, it may be due to the presence of lower adenocarcinoma in their population [27].

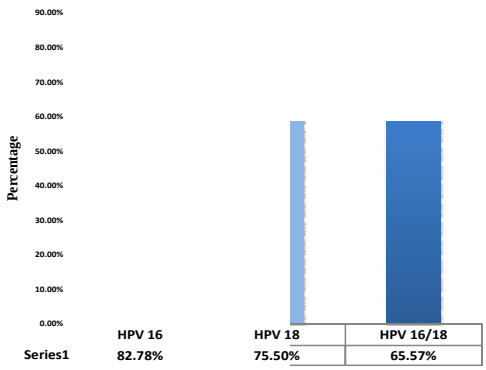

FIGURE 1: Percentage of HPV

Accordingly statistical analysis was performed with respect to age and chief complaints. In our study it was observed that many women complained of post coital bleeding and were reported to the hospital and when examined they showed signs of unhealthy cervix and bleed on touch. About 19.67% were with PCB and were statistically significant in association with age (P=13.81). According to Adam Rosenthal *et al*., and Afsaneh Tehranian *et al*., PCB may be a sign of invasive cervical cancer and bleeding may be due to delicate cervical epithelium which gets disconnected during sexual intercourse[28,29]. Whereas the other chief complaints presented by the patients in our study reported Inter menstrual bleeding P= 31.61, Menorrhagia P=150.55, Menopause P=49.07 and Post menopausal bleeding P=44.07 were statistically significant with respect to age at P<0.05. Shikha Srivastava *et al.,* reports that increased number of HPV infection in women with post menopausal bleeding was found to be associated with lower estrogen level [30].But, when white discharge with age was taken it was not statistically significant P=7.59 (fig 2).

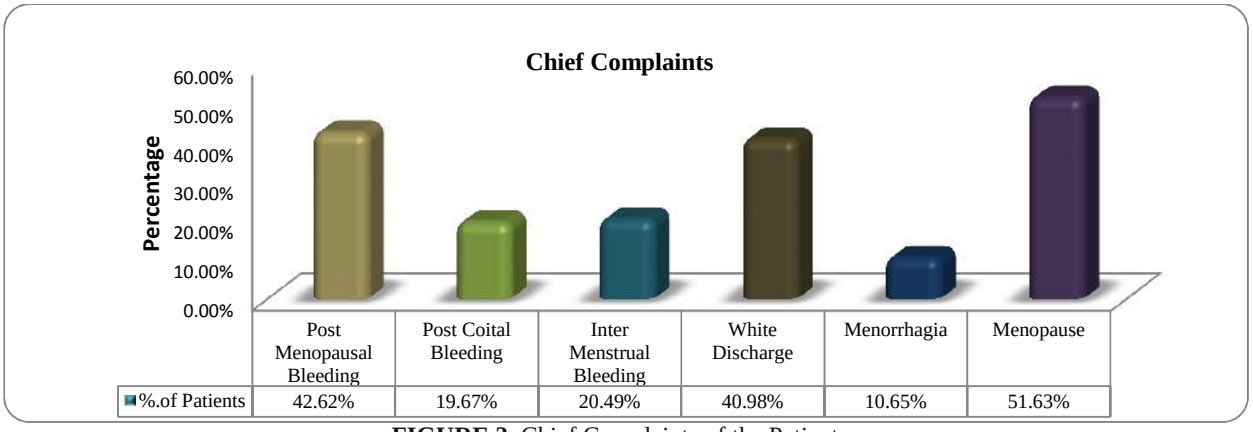

FIGURE 2: Chief Complaints of the Patients

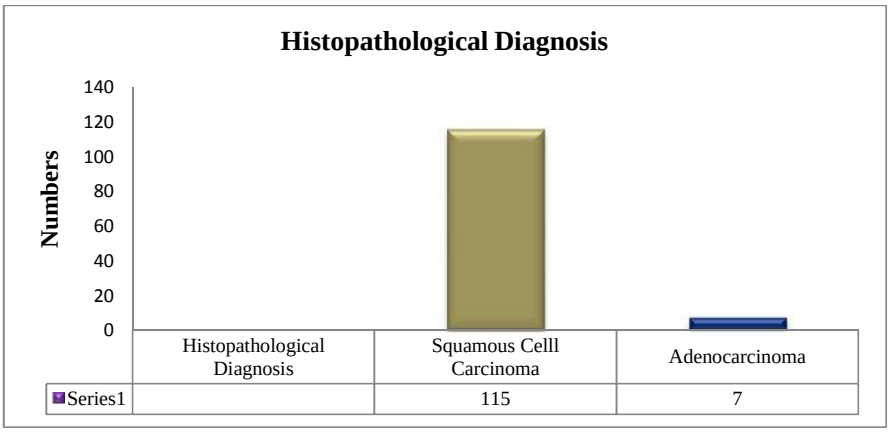

FIGURE 3: Histopathological Diagnosis

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The mean age of the patients in the study was 50 years and range from 29 to 86 years. Majority of the patients were in the range of 41 to 50 years. The age at first coitus was not considered due to cultural restraints but instead of this age at marriage was considered in our studies and it was seen that women ranged from 10 – 26 years with a mean of 16

years. All the women were married and had more than two children with single male sexual partner which is also seen by the studies done by others [31]. In the present study women who reported to the hospital were having parity with about 56.55% with 3to 4 children and 29.50% with more than 5 children. It was observed by Mishra J *et al*., that women with increased parity may be one of the cofactors for cervical cancer and because of this reason women should be screened at least once in their lifetime for the existence of premalignant lesion[23]. Our studies revealed that most of the patients were squamous cell carcinoma with about 115 (94.26%) and adenocarcinoma with 7 (5.73%) which was concurrent to the studies done in India and other parts of the world [3,32-34] (fig 3).

FIGO staging was done in our studies and it was found that women in stage III 63(51.63%) were more in number than in other stages which was also seen by Neerja Bhatla et.al.,[3]. This may be due to the delayed presenting of women to the hospital with gynecological complaints (fig 4).

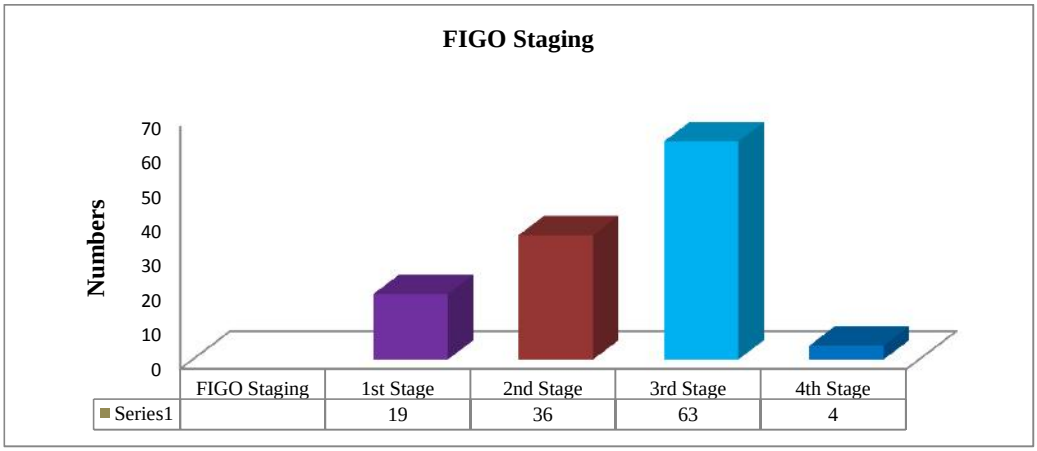

FIGURE 4: FIGO Staging

Our study strongly recommends screening for CC has to be made mandatory, so that it would be easy to identify the patients who are at the high risk end for developing CC. In the present study it was seen that most of the women reported to the hospitals were in the later stages. Diagnostic screening tools like PCR using E6 and E7 marker protein should be introduced in the hospitals and managed with adequate trained personal [35]. The screening methods should be made effective and cheap and easily available to the population.

In conclusion the highest burden of CC was found in India where there is great difficulty for effective screening[36]. Present study confirms that HPV 16 and 18 is high in this region which necessitates the screening program for women for detection of HPV which helps to identify the women at high risk of developing invasive cancer. HPV DNA amplification by PCR method which is more sensitive and specific for the detection of different HPV types is recommended to be used as a diagnostic tool. HPV typing is of utmost importance for arranging cervical cancer screening and vaccination programmes. Nowhere in India HPV screening is made mandatory to the population. Currently available HPV prophylactic vaccines targeting type 16 and 18 have high potential to reduce the burden of cervical cancer which should be made easily available to the high risk population.

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