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Detection of HPV DNA in Cervical Carcinomas after Treatment in India

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ABSTRACT Objective of this study was to evaluate the effect of treatment in eradicating HPV infection. We investigated presence of HPV DNAs in exfoliated cervical cells from cervical cancer patients before and after treatment. HPV DNA was detected by PCR using HPV consensus primers. HPV 16/18 were identified by PCR using type specific primers. HPV eradication was investigated after 3-8 months of treatment. Out of the 24 patients 4 were surgically treated and the rest by radiotherapy. HPV DNA was detected in 83.3% (20/24) of the patients before treatment. HPV types 16, 18, 16 plus 18 and HPV types other than 16/18 were demonstrated in 30%, 5%, 35%, and 30% of the patients. Following treatment 37.5% (9/24) of the patients were HPV positive. Results indicate that treatment may eradicate cervical HPV infection ($p<0.001$).

INTRODUCTION

Epidemiological evidences and laboratory studies have conclusively established association of some HPVs in developing cervical cancer (Bosch et al. 1995; Walboomers et al. 1999; zur Hausen 1994). Prevalence of HPV DNA of one or more of the greater than 20 genital types has been reported in 90-100% of cervical cancer cases (de Villiers 1989). HPV 16 and 18 are the most frequently detected types in cervical cancers (Howley 1991; zur Hausen 1991). Conflicting results are available on the relationship between specific HPV types and severity of the disease and prognosis. Some studies showed that compared to HPV 16 tumors, HPV 18 tumors are less differentiated and have more often nodal involvement (Barnes et al. 1988) and higher mortality (Walker et al. 1989). In contradiction, some studies observed no such association between HPV types and disease condition.

Radiotherapy is the most effective mode of treatment for cervical cancer. However, the factors controlling radiosensitivity of tumors are not clearly understood. Cervical carcinoma treatment is often characterized by local recurrence (3-8% for stage I to 45% for stage III) (Hunter et al. 1986). Tumor volume, stage, differentiation and lymph node involvement are possibly of prognostic significance (West 1995). Tumor proliferation index and expression of growth factors have been demonstrated to play a role in response of cervical cancer to radiotherapy (Morris GM, 1996; Pillai et al. 1998). Radiation response has also been associated with oncogene expression (Yarnold J, 1997). P53 status of tumor cells has been thought to play a great role in response to radiotherapy (Bristow et al. 1996; Kim et al. 1997). HPV may abrogate the functions of wild type p53 (Scheffner et al. 1990) and thus may influence radiosensitivity of the cervical tumors. Determination of HPV has been indicated to be useful predictor/prognostic marker (Friesland et al. 2001). However, not much study has been carried out to test efficacy of cervical cancer treatment on eradication of associated HPV infection.

In this study we investigated presence of HPV DNAs in exfoliated cervical cells from cervical cancer patients during pre and post treatment period. Our purpose was to evaluate the effect of treatment in HPV eradication and influence of the tumor stages.

MATERIALS AND METHODS

Sample Collection: Patients with invasive cervical cancer were recruited between December 1999 to June 2001 from Chittaranjan National Cancer Institute Hospital, Kolkata. The patients had histologically confirmed squamous cell carcinomas of uterine cervix and received no prior

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treatment for cancer. Stages of the disease were coded according to International Federation of Gynecology and Obstetrics. The staff of the Department of Gynecology and the Department of Radiology and Radiation Oncology analyzed staging. Only 24 patients could be followed-up for collecting cervical swabs for once 3-8 months after completion of treatment. Mean age of the patients while starting treatment was 50.33 years (range, 35-68 years).

Cervical exfoliated cells were collected in tubes containing PBS (pH 7.4), centrifuged and stored at –80^{0}C until analysis for HPV using consensus primer-based PCR (Resnick et al. 1990).

Treatment Techniques: Majority (20/24) of the follow-up patients was treated with radiation therapy alone. Remaining 4 (stage IB) requiring no postoperative radiotherapy underwent surgery. Under radiotherapy, the patients received external beam radiation of the entire pelvis and intracavitary brachytherapy irradiation (three fractions of high dose rate). Time from the start of radiation to completion was within 8 weeks. Telecobalt machine was used for external beam radiation therapy to apply a total dose of 50mgray (Gy) to the whole pelvis through antero-posterior and postero-anterior parallel opposite fields. A 2-Gy tumor dose at the midplane daily was the fraction size (five fractions every week). All patients did not receive irradiation for paraaortic lymph nodes. Intracavity irradiation was delivered with Co-60 and Ir-192. The dose of intracavity therapy was 8-Gy to Point-A weekly for 3 weeks. The first insertion was carried out when 20-Gy was administered to the pelvis. Next two insertions were performed weekly at the end of external beam radiation therapy. In other cases 3 intracavity insertions followed teletherapy. The dose rate at Point-A was more than 12-Gy per hour. The three brachytherapy fractions constituted a total dose of 24-Gy at Point-A.

DNA Isolation: Suspension of the exfoliated cervical cells in lysis buffer (10mM Tris-HCl, pH 7.5; 5mM EDTA, pH 7.9; 1% SDS) was treated with proteinase K (200 mg/ml) overnight at 37^{0}C. High molecular weight DNA was prepared by phenol extraction followed by chloroform-isoamyl alcohol (24: 1) extraction and ethanol precipitation. Concentration and quality of DNA were ascertained spectrophotometrically and by 0.8% agarose gel electrophoresis.

HPV Analysis by PCR: Overall presence of HPV DNA was demonstrated by performing PCR with consensus primers derived from the E6 open reading frames. Amplifying a fragment of 240 base pairs the PCR permitted detection of a broad spectrum of genital HPV types (Resnick et al. 1990). PCR reaction and sample preparation were carried out in two different laboratories to avoid contamination. β-globin specific primers were used as amplification control for all samples (Resnick et al, 1990). Positive and negative controls were included during each amplification reaction. To identify the specific HPV types in the HPV positive specimens further PCR was performed using HPV 16 E6 and 18 E6 specific primers (product size: HPV 16: 109bp; HPV 18: 334bp) (Miller et al. 1994).

Each amplification reaction mixture (total volume of 25 μl) contained 10 mM Tris.HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each of dNTP, 100 pmoles of each primers, approximately 500 ng of specimen DNA and 2.5 unit of thermostable DNA polymerase (Gibco BRL, USA). Amplification was carried out in the Thermal Cycler (Biorad, USA). After denaturation at 94^{0}C for 3 min, 35 cycles were used under following conditions for consensus primer mediated PCR: denaturation for 1 min at 94^{0}C, annealing at 55^{0}C for 1 min and extension at 68^{0}C for 2 min and a final 1 cycle extension at 72^{0}C for 1 min. For HPV 16/18 specific PCR the first denaturation was performed at 95^{0}C for 3 min followed by 30 cycles with denaturation at 95^{0}C for 1 min. The annealing conditions for HPV 16, 18 were 55^{0}C for 1 min and 54^{0}C for 1 min respectively. The final extension cycle for HPV 16/18 was performed at 72^{0}C for 7 min.

The PCR products (10μl) were electrophoresed in 1.8% agarose gel stained with ethidium bromide (Maniatis et al. 1982). The amplified products were identified by UV irradiation of the gels. The specimens showing PCR amplification products of the expected size were considered to be positive. Samples that were positive by PCR using consensus primers but could not be identified as HPV16 or 18 were considered as HPV X.

Following are the primer sequences used.
HPV Consensus: Forward: 5' CGG TCG GGA CCG AAA ACGG 3'
Reverse: 5' AGC ATG CGG TAT ACT GTC TC 3'
HPV 16: Forward - 5' ATT AGT GAG TAT AGA CAT TA 3'
Reverse – 5' GGC TTT TGA CAG TTA ATA CA 3'

HPV 18: Forward – 5' ACT ATG GCG CGC TTT GAG GAT CCA 3'
Reverse – 5' GGT TTC TGG CAC CGC AGG CA 3'

Statistical Analysis: Test of proportion was performed to evaluate significance of the treatment in HPV eradication.

RESULTS AND DISCUSSION

Mean ages of the HPV-positive and –negative patients were 48.35 and 60.25 years respectively.Out of the 24 patients 83.3% (20/24) showed presence of HPV DNA in their cervical swabs before start of treatment. Table 1 shows that HPV DNA types 16, 18, 16 plus 18 and 'X' (other than 16/18) were present in 30% (6/20), 5% (1/20), 35% (7/20) and 30% (6/20) of the patients respectively. But at 3 to 8 months after the end of treatment 45% (9/20) of the HPV+ patients still remained positive. In the post-treatment test majority of the patients 6/9 (66.6%) had double infection of HPV types 16 and 18 followed by HPV X type (2/9) and HPV 16 (1/9). But none had HPV 18 in the after treatment test. The 4 (4/24; 16.6%) followed up cases that were HPV negative before treatment remained so even after treatment. Thus, HPV DNA was detected in 20/24 (83.3%) patients before treatment and in 9/24 (37.5%) after the treatment. Test of proportion showed a significant ($Z=3.25$; $p<0.001$) reduction in HPV prevalence following treatment. The extent of eradication of HPV by the treatment varied with clinical stages of the patients. In stage IB it was brought down from 60% (3/5) to 20% (1/5), in IIB from 88.8% (8/9) to 44.4% (4/9) and in stage IIIB from 87.5% (7/8) to 37.5% (3/8). No particular HPV type was eradicated in preference to any other type. Out of the 6 HPV 16 positive patients 1 retained the same type, 2 became HPV 16/18 + and 3 had no HPV following treatment. HPV was eradicated by treatment from 3/7 HPV 16/18+, 4/6 HPV X+ and HPV 18 from the single patient. Thus gain in infection of additional HPV 18 was observed in 2 cases that were HPV 16+ before start of treatment.

In keeping with earlier reports (Higgins et al. 1991; Uchiyama et al. 1997) all the four HPV negative cervical carcinoma patients of this study belonged to higher age group (mean 60.25 years). Most of the patients of this study were treated by radiotherapy. However, in the United States chemotherapy administered concomitantly with radiation therapy is the currently accepted standard of care. Although a number of studies (Harima et al. 2002; Harima et al. 2001; Rantanen et al. 1998) have been carried out to analyze radiosensitivity of HPV+/HPV- sqamous carcinoma cell lines and survivability of HPV positive/negative cervical cancers treated with radiotherapy alone, very little observations have been made on the efficacy of the treatment in eradication of the virus. One group reported (Borrego et al, 2001) removal of HPV 18 (14.75% cases) and HPV X (14.75% cases) (HPV types 31, 33, 35, 39, 45, 51, 52) from all the cervical epidermoid carcinomas one month after radiotherapy. However, HPV 16 was not eradicated following treatment from 17.6% of the cases. We observed a significant ($p<0.001$) rate of HPV eradication (from 83.3% to 37.5%) in our patients, though small in numbers, 3-8 months after treatment. However, it is possible that with longer follow-up HPV could be detected in the post-treatment HPV negative cases of this study.

In our study HPV 16 was not eradicated from 50% (3/6) of the patients. HPV X was removed from 43% (3/7) cases. Following treatment few of our HPV 16 patients acquired HPV 18 additionally. This is in agreement with a recent report (Liaw et

Table 1: HPV genotypes of the patients before and after treatment with respect to clinical stages.

Clinical stages (N=24)	*HPV genotype*								*HPV persistence rate (%)*
	Before treatment (any positive=20)				*After Treatment (any positive=9)*				
	16	*18*	*16+18*	*X*	*16*	*18*	*16+18*	*X*	
IB (5)	1	-	1	1	-	-	-	1	1/3 (33)
IIB (9)	3	1	2	2	1	-	2	1	4/8 (50)
IIIB (8)	1	-	3	3	-	-	3	-	3/7 (42)
ND (2)	1	-	1	-	-	-	1	-	

ND = Stage not detected
X = HPV types other than 16/18

al. 2001) showing a general association of HPV 16 infection with greater risk of acquisition of other HPV types.

Persistence of HPV DNA in cervical cells has been reported after therapeutic conization for CIN3 (Nagai et al. 2000) and also after diathermic large loop excision treatment of CIN (Distefano et al.1998). In this study persistence of HPV infection after treatment was found to vary from 33% of patients in stage IB, 50% in stage IIB to 43% in stage IIIB. Survival rate of patients is known to decrease at advanced stage of the disease. Results of this study were indicative of greater rate of HPV persistence with reducing rate of survival of the patients. In this study HPV infection persisted in 42% (3/7) of the cases that were either HPV 16 or 18 positive before treatment. However, the persistence rate was higher (56%; 4/7) in the patients who had infections of multiple HPV types (16 and 18). This might have been possible owing to impairment of response of the patients to radiotherapy caused by the multiple type HPV infection. Eradication of the major causative infection of HPV is certainly the most effective long-term preventive measure and treatment profile may play an important role in this regard. Apart from the smaller number of study patients this investi-gation is limited by the non-existence of survival data.

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