HUMAN PAPILLOMAVIRUS AND CO-FACTORS TO CERVICAL CANCER AMONG WOMEN ATTENDED AT UNIVERSITY HOSPITAL OF UNIVERSIDADE FEDERAL FLUMINENSE

PAPILOMAVÍRUS HUMANOS E CO-FATORES PARA CÂNCER CERVICAL EM MULHERES ATENDIDAS NO HOSPITAL UNIVERSITÁRIO DA UNIVERSIDADE FEDERAL FLUMINENSE

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RESUMO

Fundamentos: Evidências laboratoriais e epidemiológicas têm demonstrado associação entre infecção por papilomavírus (HPV) e neoplasias. Tipos de HPV de alto risco tem sido relacionados a carcinoma cervical. Além da infecção por HPV, fatores adicionais têm sido envolvidos no processo de desenvolvimento de tumor. **Objetivos:** Este estudo foi empreendido para investigar infecção por HPV e co-fatores associados a câncer cervical em pacientes atendidas no Hospital Universitário Antonio Pedro (Universidade Federal Fluminense), no estado do Rio de Janeiro. **Materiais e métodos:** A amostra totalizou 35 mulheres submetidas a exame ginecológico de rotina para detecção de lesões cervicais. Dados demográficos e de risco foram obtidos por entrevista. A reação da polimerase em cadeia (PCR) foi utilizada para investigar HPV em esfregaços genitais. Os oligonucleotídeos de consenso MY09/11 foram usados para detectar DNA de HPV genérico. A tipagem de HPV foi realizada com oligonucleotídeos de seqüências do DNA do gene E6 dos HPV 6, 11, 16, 18, 31,33 e 35. **Resultados:** A maioria das pacientes era natural do estado do Rio de Janeiro, casadas ou com parceiro estável, nível de escolaridade elementar e pertenciam à classe econômica desfavorecida. Entre os fatores de risco para lesões cervicais, a maioria dos pacientes iniciou sua vida sexual entre 16 e 20 anos, tinha de 1 a 3 parceiros sexuais, não utilizava métodos anticoncepcionais, tendo de 1 a 3 paridades. Lesões cervicais incluíam ASCUS, condiloma, LSIL, HSIL e câncer. O DNA de HPV foi detectado em 94% dos casos. HPV tipo 16 foi encontrado em 100% dos carcinomas como infecção única ou associado aos tipos 18 (14%) ou 33 (8%). Observamos também que a maioria dos casos de SIL (60%) estava infectados pelo tipo 16. Entretanto, o HPV tipo 6 (baixo-risco) foi detectado em um caso de HSIL de uma paciente positiva para HIV. Os resultados mostraram uma tendência entre câncer e origem étnica ($p=0.05$). Encontramos também uma tendência de associação entre câncer e paridade ($p=0.01$). História familiar de neoplasia e abortos apresentou uma associação significativa se considerarmos $p<0.1$. Este trabalho detectou uma alta taxa de pacientes assintomáticas com lesões cervicais. **Conclusão:** Os resultados obtidos neste trabalho enfatizam a necessidade de exames ginecológicos preventivos a intervalos regulares.

Palavras-chave: HPV, lesões genitais, co-fatores para câncer cervical

ABSTRACT

Background: Evidences from laboratory and epidemiological studies have shown association between papillomavirus (HPV) infection neoplasias. High risk HPV types have been linked to cervical carcinoma. Besides HPV infection, additional factors can be involved in the process of tumor development. **Objectives:** This study was undertaken to examine HPV infection and co-factors to cervical cancer in a female population attended in Antonio Pedro Hospital (Universidade Federal Fluminennse) at Rio de Janeiro State. **Material and methods:** The sample held 35 women enrolled to routine screening for cervical lesions. Demographic and risk data were obtained by interview. A polymerase chain reaction (PCR) technique was used to investigate HPV in cervical smears. Consensus primers MY09/11 were utilized to detect generic HPV DNA. HPV typing was done with primers from the E6 gene DNA sequences of HPV 6, 11, 16, 18, 31,33, and 35. **Results:** Most of the patients were from the State of Rio de Janeiro, married or had stable partner, had elementary school and were from low economic class. About risk factors for cervical lesions, most of them had begun their sexual life between 16-20 years old, have had 1-3 sexual partners during their lives, have used no contraceptive methods, had 1-3 parities. Cervical lesions included ASCUS, condyloma, LSIL, HSIL and cancer. HPV DNA was detect in 94% of the cases. HPV type 16 was found in 100% of the carcinomas alone or associated to HPV type 18 (14%) or 33 (8%). It was also found that most of the SIL (60%) were infected by HPV type 16. However, the HPV type 6 (low risk) was detected in a HSIL of an HIV positive woman. It was noted a trend between cancer prevalence and ethnic origin (0.05). We also found a trend of association between cancer and parity ($p=0.01$). History of familiar neoplasia and abortions also had a significance if we consider $p<0.1$. This work detected a high rate of asymptomatic patients carrying neoplasic lesions. **Conclusion:** The data obtained in this work emphasize the need of gynaecologic preventive exams at regular intervals.

Keywords: HPV, genital lesions, co-factors to cervical cancer

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INTRODUCTION

Breast cancer is the highest cause of incidence and mortality among female malignancies, and the cervical cancer have declined in developed countries. However, this neoplasm promotes high rates of sickness and mortality in developing countries included Brazil. Around 40000 cases of cervical cancer arises per year among Brazilian women.

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LOPES et al. studying the behavior of the Brazilian women in face to prevention of cervical cancer verified that most of the people presented lack of preventive exams[1]. Uterine cervix cancer and not specified uterine part cancer were the second rate of underlying causes of death owing to neoplasms for women aged 30-49 in the state of São Paulo[2].

Evidences from laboratory and epidemiological studies have shown association between papillomavirus (HPV) infection and both cervical cancer and precancerous neoplasias[3,4]. High risk HPV types as HPV 16 and 18 have been strongly linked to cervical carcinoma[5]. Besides HPV infection, additional factors can be involved in the process of tumor development. Epidemiological studies have also shown relationship between cervical neoplasia and others potential risk factors as sexual behaviour, genetic predispositions, immune status, smoking, diet, income status[6]. Few studies have examined the role of demographic factors as racial/ethnic differences and intraepithelial lesions and HPV infection[7]. Geographic disparities were also found for HPV infection and cervical intraepithelial neoplasias (CIN)[8].

In this current study, we conduct a small group of women who underwent routine Papanicolau test to assess the association between HPV infection, cervical cancer and demographic and risk factors.

MATERIAL AND METHODS

Patients

In this wok we have studied 35 women attending at Hospital Universitário Antônio Pedro of Universidade Federal Fluminense, Rio de Janeiro between April 2000 and December 2001. Colpocitology test screening was performed at the first or subsequent visit to the clinic. For women with abnormal cervical cytology biopsies were performed.

Interviews

Demographic data (sex, age, place of birth, ethnic origin, civil status, education, and socio-economic conditions) and risk factors (age at first intercourse, number of labours, abortions, smoking, drug users, life time number of sexual partners, sexually transmitted disease history, familiar history of neoplasias, colpocitology, histology, immunossupressors, HIV infection and contraceptive and reproductive history) were obtained from patients by an interview using a structured questionnaire. All patients gave written informed consent.

Materials

Cervical smears containing ectocervical and endocervical cells were taken from each patient, placed in TE buffer (TRIS 10 mM pH 7.4, EDTA 1mM) and stored at -20^{0}C.

Colpocitology

The cases were classified as Normal, ASCUS (atypical squamous cells of undetermined significance), HPV infection, low grade squamous intraepithelial lesions (LSIL -CIN I), high grade squamous intraepithelial lesions (HSIL - CIN II e III), carcinoma "in situ" and squamous invasive carcinoma (CA).

DNA extraction

Samples were incubated for 4 hours at 50^{0}C in 200 ml of digestion buffer (10mM TRIS-Hydrochloric acid pH 8.3, 1mM EDTA pH 8.0, 0,5% Tween 20, proteinase K, 400 mg/ml of final concentration). After they were extracted with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated with one tenth volume of 0,3M sodium acetate and three volumes of 100% ice cold ethanol, washed with 70% ethanol, air dried and suspended in 50 ml of sterile water.

PCR amplification of generic HPV

Consensus primers MY09/11 which amplify 450 bp DNA sequences within the L1 region of HPV were used to detect generic HPV DNA. Amplification was carried out in 50 ml reaction mixture (1X PCR buffer, 200 mM dNTPs, 1,5mM MgCl , 50 pmol of each primer, 0,25U unit of Taq polymerase, and 5^2 ml of sample) with 35 cycles of amplification. Each cycle included a denaturation step at 94^{0}C for 1 minute, an annealing step at 55^{0}C for 2 minutes, and a chain elongation step at 72^{0}C for 2 minutes using DNA Thermal Cycler (Pekin Elmer, CETUS). The b-actine primers (0,1 pmol each), which amplify a 330 bp region of the human DNA was used as internal control. PCR products were analysed on 1,3% agarose gel with ethidium bromide staining for visualisation of DNA under ultraviolet light and their PM determined by comparison with a 100bp DNA ladder.

HPV typing

HPV typing was done by PCR amplification with primers from the E6 gene DNA sequences of HPV 6, 11, 16, 18, 31,33, and 35 (Table 1). PCR reactions included a denaturating step at 94^{0}C for 30 seconds, 60^{0}C for 1 minute, and 72^{0}C for 1 minute. Negative controls for background contamination no added DNA template. The PCR run was completed by extension for 10 minutes at 72^{0}C.

Statistical analysis

To evaluate if the selected factors affect the probability of cervical cancer in a small sample, tests for trend were performed. Data were analysed using EPInfo 2000 statistical software package (Center for Disease Control and Prevention, Atlanta, Georgia, EUA, 2000). The Chi-squared test assessed the trend between variables and carcinoma (in situ and squamous) Probability values smaller than 0,05 were considered significant.

RESULTS

Description of the sample

The demographic finds of the sample are listed in table 2. The age range was 18-68 years with a average of 37.8 years, standard deviation, 11.9 .

Most of the patients (74%) were from the State of Rio de Janeiro. Among them, 43% were white, 20% had black origin and 34% were mulattos. The analysis of the relationship status revealed that 34% were single, 49% were married or had stable partner, 6% were divorcee and 11% were widower. Considering the education level, 3% were illiterates; 53% had elementary school (completed or not); 31% had high school (completed or not); and 3% had college (completed or not). At the time of the survey, 49% had familiar income about one or two living wages, 29% had from 3 to 5 living wages, 8% had from 6 to 10 living wages and 8% had up 10 living wages. One living wage is about 80 dollars.

According to cytological diagnosis we have found 4 normal cases, 2 ASCUS, 5 HPV infection, 9 LSIL, 7 HSIL and 8 carcinomas. Cervical cancer and demographic variables

are given in the Table 2. We have observed a trend between cancer prevalence and ethnic origin. The highest cancer prevalence was found in the black women group (p=0.05).

The risk factors data are showed in the Table 3. - Among the patients, 34% had begun their sexual life between under 16 years old, 54% between 16-20 years old, 8% between 21-25 years old and 2% up to 25 years old; 57% have had 1-3 sexual partners during their lives, 28% have had 3-4 sexual partners and 14% have had multiple sexual partners; 18% have used oral contraceptive methods, 14% have used condom, 28% have made Fallopian tube attachment, 38% have used no methods and 2% have no sexual relations; 8% have no parity, 55% have had 1-3 parities, 31% have had 4-7 and 6% did not answered; 43% never done abortion, 40% have done 1-2 abortions, 11% have done up to 3 and 6% did not answered; 14% have had menarche between 14 years old, 34% have had between 12-13 years old, 45% between 14-15 years old, 2% up to 16 years old and 2% did not answered; 31% were smoker and 69% were non smoker; 14% were drunker, 77% were non drunker and 8% did not answered; 20% had have others sexual transmitted disease than HPV and 80% no had; 45% have history of familiar neoplasia , 41% did not have and 14% did not answered. Risk factors data suggest a trend of association between cancer and parity (p=0.01). History of familiar neoplasia and abortions also had a trend of significance if we considerer p<0,1.

The clinical history, cytology and histology diagnosis are listed in the table 4. The study revealed fourteen women without complaint covering several kinds of cervical lesions including three HSIL and one cancer case.

Detection of HPV DNA

The results were summarised in the Tables 5 and 6. The sample had a high rate of HPV infection. Ninety fourth per cent of the patients were positive for the presence of HPV genome when they were tested to L1 and E6 HPV. One carcinoma were negative to L1 primers but positive to 16 and 18 E6 primers. HPV type 16 was found in 100% of the carcinomas or alone or associated to HPV type 18 (14%) or 33 (8%). It was also found that most of the SIL (60%) were infected by HPV type 16. Among six cases of condyloma, four were associated to SIL and all of them were positive to HPV as showed in the table 5. Four positive HPV patients (11%) were also HIV positive and the HPV type 6 was detected in a HSIL of an HIV positive woman.

DISCUSSION

Infection with high-risk HPV type is frequent among sexually active women, with incidence ranging from 15 to 40%[9] . However, the majority of the infections are found to be transient because most of individuals develop an specific immune response[10]. When the infection persists precancer lesions can be developed. About 1% of the population present genital warts and 4% of women have SIL. High grade SIL observed in women aged 35-40 years are at high risk of progression toward cancer [6].

Our findings support a strong association between HPV infection and cervical diseases. HPV 16 was the most frequent type in benign as well as in premalignant or malignant cases. The exception occurred in a HIV positive woman, carrying a LSIL and infected by HPV type 6. HPV is considered a opportunistic infection in these patients. The deep failure of the immune status linked to HIV patients can increase the development of the premalignant neoplasia. The association between HIV and HPV carried the Center for Disease Control to consider the uterine cancer as an AIDS disease[11]. All of the carcinomas had HPV type 16 infection. The use of two sets of primers increased HPV detection in cervical tumors, elucidating its etiology. Walboomers JM, Jacob MV, Manos MM et al., 2001[12], in a review article relates HPV infection in 99% of the invasive cervical cancer cases.

However, if HPV infection leading the process of tumor development, it is not enough. Co-factors try for this process. Despite the small number of cases, our results show some of these variables affecting the cervical cancer incidence.

According to sociodemographic data, most of the patients were from lower economic class. In comparison to demographic characteristics and cervical carcinoma, we have observed that black women had a higher incidence of cancer than in white women. This data showed to be independent of income class. It is worth to observe that 14% of the women had HPV 18, a frequent type found in Rio de Janeiro[13]. Aleixo Neto (1991)[14], have reported in a review paper that the incidence of cervical cancer in United States,1978, was 6.8/100,000 among white women and 14.7/100,000 among black women.

Among risk factors analysed, we have also found a significant trend between 4-7 parities and cancer incidence. Three patients of this group were black. The small number of cases do not allow a statistical treatment, hence we were not able to associate these variables. The number of parities is considered a risk factor to cervical cancer [15]. Trauma to the cervix or hormonal effects could increase the risk of malignant development. Abortion and familiar neoplasia could have significance for cancer if we consider p<0.1. Genetic predisposition to cervical neoplasia have been described[16]. Recent studies show that the development of the cervical cancer can be associated to HLA alleles[17,18]. but we not found reports which associates number of abortions to cervical cancer. Although sexual history and smoking are established co-factors to cervical neoplasia, we did not found any trend to these variables.

One important and preoccupant data obtained was the high rate of asymptomatic cases carrying neoplasic lesions, as showed in Table 4. The presence of three HSIL and one carcinoma emphasize the need of gynaecologic preventive exams at regular intervals. A remarkable finding, the asymptomatic woman with carcinoma in situ were affected by several risk variables: HPV type 16 and 18 infection, black ethinia, 40 years old, smoker, multiple partners, six parities, two abortions and familiar neoplasia. Besides variables out of control, there were co-factors preventable by educational and health policies.

CONCLUSION

Cervical cancer is a preventable disease. Strategies to prevent it include the screening of the female population trough Papanicolao test , HPV testing, close management and follow-up of women with precancerous lesions. Despite

the limitations of this work, we have shown a sample with high incidence of HPV infection associated to SIL, condyloma and carcinoma. All the patients have been treated in the most of the cases and they have came back to the hospital to be submitted a regular follow-up .

Table 1 - Promers sequence of specific E6 HPV types:

DNA HPV	Primers
6 P1/P2	CAC CAT AAG GTC CTG TTT/ GAA CCG CGC CTT GGT TAG
11P1/P2	CGC AGA GAT ATA TGC ATA TG/ AGT TCT AAG CAA CAG GCA CA
16P1/P2	CC AGA AAG TTA CCA CAG/ TAC TAT GCA TAA ATC CCG
18P1/P2	GAA ACC GTT GAA TCC AGC/ GTT CCT GTC GTC CTC GGT
31P1/P2	GAC CTC GGA AAT TGC ATC/ TGT TTC TGT TAA CTG ACC
33P1/P2	GTA TAT AGA GAG GGA AAT/TAA AGG TTT TTT AAC TGT
35P1/P2	ACA AGA ATT ACA GCG GAG/TAA CTG TTT GTT GCA TTG

Table 2 - Demographic details and cytological diagnosis of the sample studied (N=35)

	N	%	Normal n=4	ASCUS n=2	HPV n=5	LSIL n=9	HSIL n=7	CA n=8	p*value
Age group									
<20	2	6	0	0	1	0	1	0	0.28
20-29	6	17	2	1	1	2	0	0	
30-39	12	34	0	1	1	4	3	3	
40-49	9	26	0	0	2	3	2	2	
50-59	5	14	2	0	0	1	1	1	
>60	1	3	0	0	0	0	0	1	
From Rio de Janeiro									
Yes	25	71	3	2	2	6	5	5	0.61
No	9	27	0	0	3	3	2	3	
Unknown	1	22	1	0	0	0	0	0	
Ethnic origin									
White	15	43	2	1	2	5	4	1	
Black	7	20	0	0	2	0	0	5	0.05
Mulattos	12	34	2	1	0	4	3	2	
Others	1	3	0	0	1	0	0	0	
Civil status									
Single	12	34	1	1	2	2	2	4	0.22
Married	17	49	2	1	3	4	5	2	
Divorcee	2	6	1	0	0	1	0	0	
Widower	4	11	0	0	0	2	0	2	
Education level									
Illiterate	1	3	0	0	0	0	1	0	0.21
Elementary school	19	53	3	1	2	3	5	5	
High school	13	31	1	1	3	5	1	2	
College	1	3	0	0	0	1	0	0	
Unknown	1		0	0	0	0	0	1	
Familiar income[a]									
1-2	19	49	4	0	2	4	4	5	0.4
3-5	11	29	0	2	1	3	3	2	
6-10	3	8	0	0	1	2	0	0	
>10	0	8	0	0	0	0	0	0	
Unknown	2	6	0	0	1	0	0	1	

p* value between CA and variables a: number of minimum salaries (US 80 each) received per month; N: normal; One minimum salary is equivalent to $80,00 per month

Table 3 - Sexual history and risk factors of the sample studied and cervical lesion

	N=35	%	Normal n=4	ASCUS n=2	HPV n=5	LSIL n=9	HSIL n=7	CA n=8	p-value
Age of sexual debut									
<16	12	34	0	0	2	3	4	3	0.8
16-20	19	54	4	2	2	4	2	5	
21-25	3	8	0	0	1	1	1	0	
>25	1	2	0	0	0	1	0	0	
Number of lifetime sexual partners									
1-2	20	57	2	1	3	7	4	3	0.9
3-4	10	28	2	1	2	1	1	3	
Multiple	5	14	0	0	0	1	2	2	
Methods anticontraceptives									
OC	6	18	1	0	2	1	2	0	0.7
Condom	5	14	1	1	2	0	0	1	
LT	10	28	0	0	0	4	3	3	
No methods	13	38	2	1	1	3	2	4	
No relations	1	2	0	0	0	1	0	0	
Parity									
0	3	8	1	0	1	1	0	0	
1-3	19	55	1	2	2	7	5	2	
4-7	11	31	2	0	1	1	2	6	0.01
Unknown	2	6			2				
Abortions									
0	15	43	1	0	5	4	3	2	0.08
1-2	14	40	1	2	0	5	3	3	
=>3	4	11	0	0	0	0	1	3	
Unknown	2	6	2						
Age of menarche									
10-11	5	14	1	0	1	1	1	1	0.6
12-13	12	34	1	0	3	2	2	4	
14-15	16	45	2	2	0	6	3	3	
16	1	2	0	0	0	0	1	0	
Unknown	1	2	0	0	1	0	0	0	
Smoking									
Yes	11	31	1	0	2	3	2	3	0.8
No	24	69	3	2	3	6	5	5	
Drunker									
Yes	5	14	1	0	0	2	0	2	0.2
No	27	77	3	2	3	7	6	6	
Unknown	3	8	0	0	2	0	1	0	
Others STD									
Yes	7	20	0	0	2	3	2	0	0.2
No	28	80	4	2	3	6	5	8	
Family neoplasia history									
Yes	16	45	2	2	2	3	3	5	0.07
No	14	41	2	1	1	6	2	2	
Unknown	5	14	0	0	2	0	2	1	

Table 4 - Clinical and cytological/histological findings

Clinical findings	N	Normal	ASCUS	HPV	LSIL	HSIL	CA
Asymptomatic	14	2	2	1	5	3	1**
Condyloma	6	1*		2	2	1	
Vegetant lesion	8					1	7
HIV	4			2	1	1	
Others diseases	3	1			1	1	

*Condyloma perianal **in situ carcinoma

Table 5 - Clinic findings, cytological/histological and HPV detection of asymptomatic patients

Patient number	Clinic findings	Colpocitology	Biopsy	HPV	L1	E6	Type
1	Asymptomatic	ASCUS	CIN II	No	No	No	Missing
4	Asymptomatic	Normal	HPV	No	No	No	16,18
5	Asymptomatic	ASCUS	HPV	Yes	Yes	Yes	Missing
8	Asymptomatic	LSIL	CIN I/HPV	Yes	Yes	Yes	6
9	AsymptomaTic	LSIL	Hyperplasia	Yes	Yes	Yes	6
11	Asymptoma'ic	HPV	Missing	Aes	Yes	yes	16,18
12	Asymptomatac	Normal	HPV	Yes	Yes	Yew	16
14	Asymptomatic	HSAL	CIN III	Yes	Yas	Yes	6,16
18	Asymptomqtic	LSID/HPV	Missing	Yes	Yes	Yes	16
29	Asimptomatic	LSIL	Conization-NIC III	Yes	No	Yes	16
32	Asymptomatic	LSIL	HTV	Yes	Yas	Yes	16,18
38	Asymptomatic	CA in situ	CA in situ	Yes	Yes	Missing	16,18

Table 6 - Clinic findings, cytological/histological and HPV detection of symptomatic patients

7	Condyloma	LSIL	HPV	Yes	Yes	Yes	6,33
13	Condylome perianal	Normal	Condylgma acuminate	Yes	Yes	Yes	6,16
20	Condyloma	LSIL/HPV	Missing	Yec	Yes	Yew	16
24	Condyloma	HPV	Cgndyloma acuminate	Yes	Yes	Yes	6,11
31	Condyloma	HSIL	HSIL (CINII)	Yes	yes	Yes	Iissing
35	Condiloma	HPv	Missing	Yes	Yes	Missing	16,18
2	Vegetant lesion	HSIL	CIN III	Yes	Yes	Aes	16
7	Vegetaft lesion	Squamous CA	Squamous CA	Yes	No	Yes	16,18
15	Vegetant lesion	AdenoCA in situ	CIN III/HPV	Yes	Yes	Yes	16,33
21	Vegetant lesion	AdenoCA	AdenoCA/ HPV	Yes	Yes	Yes	16
23	Vegetant lesion	Squamous CA	CIN III	Yes	No	Yes	16
28	Vegetant lesion	Squamous CA	Squamous CA /HPV	Yes	Yes	Yes	16
30	Vegetant lesion	Squamous CA	Squamous CA	Yes	No	Yes	16
33	Vegetant lesion	CA in situ	CA in situ	Yes	Yes	Yes	16
19	HIV	LSIL/HPV	Missing	Yes	Yes	Yes	33
6	HIV/Herpes	HSIL	HPV/Herpes	Yes	Yes	Yes	6
22	HIV	HPV	Missing	Yes	Yes	Yes	16
36	HIV/HPV	HPV	Missing	Missing	Missing	Missing	Missing
17	Diabetis	HSIL	CIN not classified	Yes	Yes	Yes	16
26	Renal ins.	LSIL/HPV	LSIL/HPV	Yes	Yes	Yes	16
27	Conisation/HPV	Normal	LSIL/HPV	Yes	No	Yes	16

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