Detection of *ras* gene mutations and HPV in lesions of the human female reproductive tract

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Abstract. Compatible with the epidemiology and natural history of cervical cancer and with experimental findings that HPV-immortalized nontumorigenic cells are similar to cervical carcinoma cells, in terms of the quantity and type of viral RNA expressed, it is believed that additional cellular genetic events are necessary for tumorigenic conversion. In the current study we detected codon 12 point mutations of the K-*ras* oncogene with an incidence of 28.2% in malignant lesions of the cervix, as well as HPV18 at a higher rate than HPV16 (30.2% vs 27.9%) in genital lesions, by PCR and RFLP analysis. Codon 12 point mutations of K-, H- and N-*ras* were also found in benign lesions of the cervix, in endometrial and in ovarian carcinomas, although at a lower frequency. It is suggested that the mutationally activated *ras* oncogenes cooperate with HPV in the early stages of carcinogenesis of the human female reproductive tract.

Introduction

Human papilloma viruses (HPVs) are strongly implicated in the genesis of human cervical carcinoma, anal squamous cell carcinoma, squamous cell carcinoma of the skin and isolated cases of squamous cell carcinoma of the upper respiratory tract (1,2). At least 24 of more than 65 genotypes of HPV are associated with anogenital lesions and they can be subdivided into two groups, the 'low risk' HPVs such as HPV6 and HPV11 and the 'high risk' HPVs such as HPV16 and HPV18. HPV DNA has been identified in precancerous and neoplastic lesions of the uterine cervix in over 84% of patients examined (3).

At least two early genes, E6 and E7, are consistently expressed in HPV-associated cervical cancers and their derived cell lines. E6 and E7 genes bind to the cellular p53 and Rb tumor suppressor proteins, respectively (4-6) and are sufficient for *in vitro* cellular immortalization (7,8). In contrast to fully transformed cervical carcinoma cells, HPV-immortalized keratinocytes are nontumorigenic in nude mice (9).

As is the case for other DNA tumor viruses, the development of HPV-associated cancer is presumed to be a multistep process. Since HPV16 and HPV18 are able to immortalize primary keratinocytes, but they are not sufficient, except in rare cases, to engender a full tumorigenic conversion (10), it has been suggested that activation of cellular oncogenes is necessary for the progression of cervical cancer. Activation of c-*myc* and H-*ras* appears to be quite common in cervical cancers, and overexpression of c-*myc* is associated with poor prognosis (11,12). Furthermore, it has been demonstrated that a *ras* gene can induce tumorigenic conversion of HPV-immortalized cervical keratinocytes (13,14) indicating a cooperative effect between the *ras* and E6/E7 genes in cellular transformation.

Additionally, the observation that overexpression of HPV18 E6, c-*myc* or activated H-*ras* can partially overcome the growth inhibitory effect of wild-type p53 in NIH3T3 cells, suggests that the cooperation between HPV E6 and these cellular oncogenes is necessary to completely overcome the anti-oncogenic reaction of p53 in the development of these tumors (15). It has also been demonstrated that the rapid ubiquitin-dependent degradation of p53 through the E6-E6 associated protein complex (E6-E6-AP) requires additional cellular factors, present only in specific tissues or at certain phases during cell growth or differentiation (16).

Alterations in *fos* expression, which appear to be an important facet of epidermal differentiation *in vivo*, could also contribute to the malignant progression of HPV immortalized cells (17).

Activated *ras* genes by point mutations have been reported in cervical cancer, although at low frequency (18), while in endometrial carcinomas the frequency was significantly higher (18-20). Mutations of K-*ras* oncogene have also been reported in human mucinous ovarian tumors (21).

In the current study the presence of codon 12 point mutations of K-, H- and N-*ras* genes, as well as detection and typing of the human papillomavirus was examined in 112

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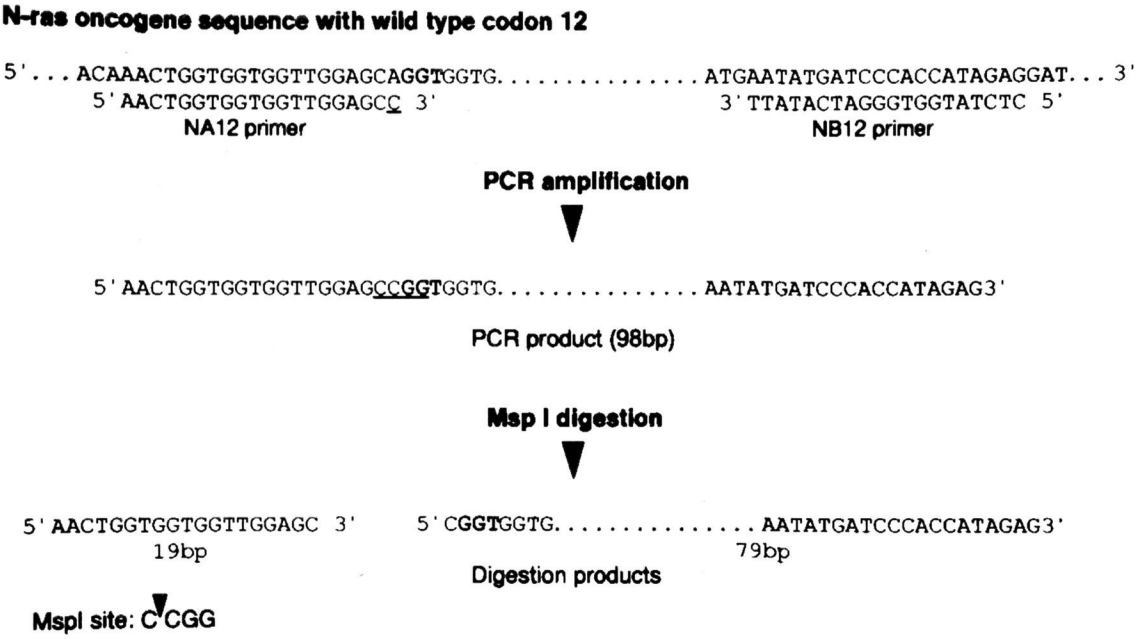

Figure 1. N-*ras* codon 12 PCR-RFLP analysis. The PCR amplification product (98bp) was digested with the restriction endonuclease MspI. Samples containing the normal codon 12 produced a 79 and a 19bp fragment. Samples containing mutant codon 12 (at 1st and 2nd position) remained undigested (98bp).

cases of genital lesions. Mutationally activated *ras* genes were found in cervical, endometrial and ovarian lesions, with the highest incidence occuring in cervical malignant lesions (28.2% of K-*ras* mutations). HPV was found in cervical and endometrial lesions, with a higher rate in malignant cervical lesions (76.9%). HPV18 was detected at a higher rate than HPV16 (30.2% vs 27.9%) in genital lesions.

Materials and methods

Patients and specimens. Tissue specimens were obtained from 112 patients with genital lesions, treated at the Department of Obstetrics and Gynecology, Medical School, University of Crete, Heraklion and the Department of Obstetrics and Gynecology, Medical School, University of Ioannina, Greece. Hematoxylin-eosin stained sections from all paraffin blocks were reviewed to reconfirm the diagnosis, the tumor type and differentiation and representative blocks (one per case) were selected for further analysis.

DNA extraction. Five or six 10 μm thick sections from formalin fixed, paraffin-embedded tissues were lysed in 400 μl digestion buffer, containing 100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS pH 8.0 and 0.1 mg/ml proteinase K. Samples were incubated for 2 h at 60°C. Fresh proteinase K was added and the incubation was continued for another 2 h. The samples were then extracted twice with phenol/ chloroform and once with chloroform. DNA was precipitated with the addition of 25 μl 5 M NaCl and 1 ml ethanol, recovered with centrifugation for 15 min at 4°C, washed once with cold 70% ethanol and resuspended in 20 μl double distilled water.

Oligonucleotide primers and PCR amplification. The oligonucleotides used for K-*ras* and H-*ras* codon 12 have been previously described (22). For N-*ras* codon 12 we used the oligonucleotides: NA12: 5' AACTGGTGGTGGT TGGAGCC 3' and NB12: 5' CTCTATGGTGGGATCA TATT 3' (Fig. 1). For the detection and distinction of the HPV the general primers GP5 and GP6 (23) and specific primers to amplify each virus type HPV11, 16, 18 and 33 (24) were used. *Taq* polymerase was purchased from Advanced Biotechnology. Two μl of the extracted DNA of each sample were amplified in a volume of 50 μl under the following conditions:

K-ras amplification. The reaction solution contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 3.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of each dNTP, 1 μM of each primer and 1.25 U *Taq* polymerase. The mixture was heated for 1 min at 95°C and then subjected to 40 cycles of amplification. Each cycle included a denaturation step at 94°C for 50 sec, an annealing step at 58°C for 45 sec and an elongation step at 72°C for 50 sec, increasing the elongation time 1 sec per cycle.

H-ras amplification. The reaction solution contained 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween, 200 μM of each dNTP, 0.5 μM of each primer and 1.25 U *Taq* polymerase. The mixture was heated for 1 min at 95°C, and then subjected to 40 cycles of amplification. Each cycle included a denaturation step at 94°C for 50 sec, an annealing step at 62°C for 40 sec and an elongation step at 72°C for 50 sec, increasing the elongation time 1 sec per cycle.

N-ras amplification. The reaction solution contained 20 mM $(NH_4)_2SO_4$, 2.25 mM $MgCl_2$, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween, 200 μM of each dNTP, 0.75 μM of each primer and 1.25 U *Taq* polymerase. The mixture was heated for 1 min at 95°C, and then subjected to 40 cycles of amplification. Each cycle included a denaturation step at 94°C for 1 min, an

annealing step at 58°C for 45 sec and an elongation step at 72°C for 25 sec, increasing the elongation time 1 sec per cycle.

HPV amplification. The reaction solution, using the general primers GP5 and GP6, contained 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween, 200 µM of each dNTP, 0.75 µM of each primer and 1.25 U *Taq* polymerase. The mixture was heated for 1 min at 95°C, and then subjected to 40 cycles of amplification. Each cycle included a denaturation step at 94°C for 50 sec, an annealing step at 52°C for 50 sec and an elongation step at 72°C for 45 sec, increasing the elongation time 1 sec per cycle.

Multiplex PCR was employed for the distinction of HPV types, where four pairs of primers were used stimultaneously (for HPV types 11, 16, 18 and 33), with each virus type giving different length of amplified DNA. The reaction solution, using the specific set of primers contained 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween, 250 µM of each dNTP, 0.5 µM of each primer and 1.25 U *Taq* polymerase. The mixture was heated for 1 min at 95°C, and then subjected to 35 cycles of amplification. Each cycle included a denaturation step at 94°C for 1 min, an annealing step at 56°C for 50 sec and an elongation step at 72°C for 50 sec, increasing the elongation time 1 sec per cycle. To establish type specificity of primer-directed ampification, each set of primers was tested with template plasmid DNA of the five HPV types 6b, 11, 16, 18 and 33.

RFLP analysis. K-ras: 10-20 µl aliquots of the amplification products were digested for 3 h with 40 U BstNI.

H-ras or N-ras. 10-20 µl aliquots of the amplification products were digested O/N with 40 U MspI.

Digestion products were electrophoresed through an 8% polyacrylamide gel, or a 4% agarose gel.

HPV-general primers. The type distinction using the specific primers was reconfirmed with digestion of the amplification products, using the general primers, with 50 U of RsaI giving a different pattern for each type. Digestion products were electrophoresed through a 10% polyacrylamide gel. As control the amplified product of plasmid DNA of the HPV types 6b, 11, 16, 18 and 33 were used.

Gels were stained with ethidium bromide and photographed on a UV light transilluminator. Enzymes were supplied by New England Biolabs and the conditions followed for digestions were those recommended by the supplier. Incubation temperatures were 60°C for BstNI and 37°C for MspI and RsaI.

Statistical analysis. Statistical analysis was performed on a Macintosh computer with the help of STATWORK (version 1.2) software.

Results

After reviewing the selected tissue sections of 112 samples, the histological diagnosis, tumor classification and differentiation was as follows: There were 37 cases of cervicitis, 10 cervical intraepithelial neoplasias (CIN I: 3 cases, CIN II: 1 case, CIN II-III: 1 case, CIN III: 5 cases), 7 cervical adenocarcinomas (well differentiated: 2 cases, moderately: 2 cases, poorly: 3 cases), 1 *in situ* squamous cell cervical carcinoma, 29 squamous cell cervical carcinomas (well differentiated: 2 cases, moderately: 23 cases, poorly: 4 cases), 1 undifferentiated clear cell carcinoma, 14 endometrial adenocarcinomas (well differentiated: 7 cases, moderately: 5 cases, poorly: 1 case, Grade IV: 1 case), 1 poorly differentiated clear cell endometrial carcinoma, 1 leiomyosarcoma of the uterus, 5 ovarian adenocarcinomas (poorly differentiated: 2 cases), 1 moderately differentiated serous ovarian cystadenocarcinoma, 1 borderline mucinous ovarian tumor, 1 Krukenberg tumor, 2 squamous cell carcinomas of the vulva (moderately differentiated: 1 case).

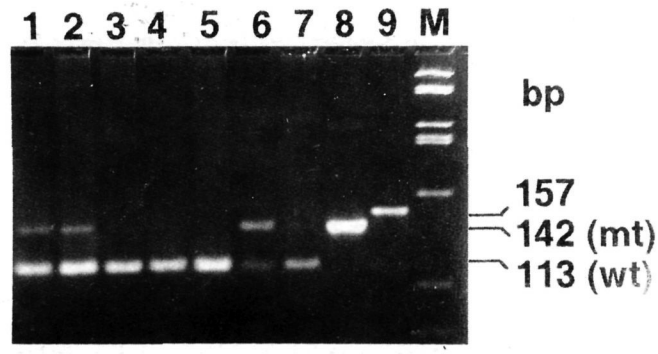

Figure 2. K-*ras* amplification products (157bp) were digested with the restriction endonuclease BstNI and electrophoresed through a 4% agarose gel. Lanes 1,2,6: positive samples, lanes 3,4,5,7: negative samples (113bp), lane 8: positive control SW480 cell line (142bp), lane 9: undigested PCR product, lane M: molecular weight marker pUC18/HaeIII.

In the current study we examined the presence of point mutations in codon 12 of K-, H- and N-*ras* genes and performed detection and distinction of the human papillomavirus in 112 cases of genital lesions.

Sixteen out of the 112 samples were found to carry a point mutation in codon 12 of K-*ras* (Fig. 2), 9 of H-*ras* (Fig. 3), while only 4 of N-*ras* gene (Fig. 4). Our study was limited to codon 12 of the K-*ras* gene, since mutations preferentially occur at this codon (25). The K-*ras* mutations were found in 2 out of 37 (5.4%) patients with cervicitis, in 1 out of 10 (10%) with CIN II, in 2 out of 7 (28.6%) with well differentiated cervical adenocarcinoma, in 1 patient with *in situ* squamous cell carcinoma, in 7 out of 29 (24.1%) with squamous cell carcinoma of the cervix (6 cases of moderately and 1 case of poorly differentiated), in 1 patient with moderately differentiated mucoepidermoid carcinoma of the cervix, in 1 out of 14 (7.1%) with a moderately differentiated endometrial adenocarcinoma and 1 out of 5 (20%) patients with a poorly differentiated ovarian adenocarcinoma. The H-*ras* mutations were found in 2 out of 37 (5.4%) patients with cervicitis, in 2 out of 10 (20%) with CIN II and CIN III, in 1 out of 7 (14.3%) with a well differentiated cervical adenocarcinoma, in 2 out of 31 (6.5%) with moderately differentiated squamous cell cervical carcinoma and in 2 out of 14 (14.3%) with moderately differentiated endometrial adenocarcinoma. The N-*ras* mutations were found in 1 out of 37 (2.7%) patients with cervicitis, in 1 patient of *in situ* squamous cell carcinoma of the cervix and in 2 out of 29

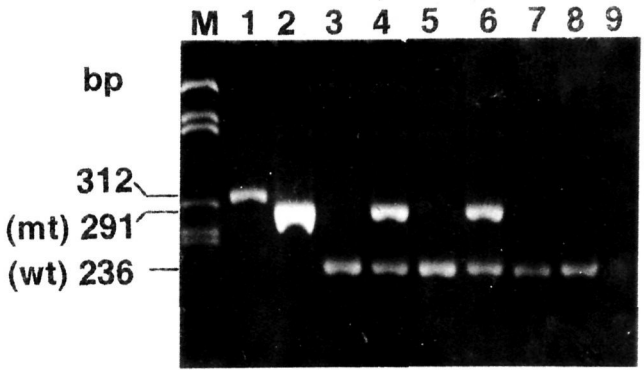

Figure 3. H-*ras* amplification products (312bp) were digested with the restriction endonuclease MspI and electrophoresed through a 4% agarose gel. Lane M: molecular weight marker pUC18/HaeIII, lane 1: undigested PCR product, lane 2: positive control EJ cell line (291bp), lanes 3,5,7,8,9 negative samples, lanes 4,6: positive samples (236bp).

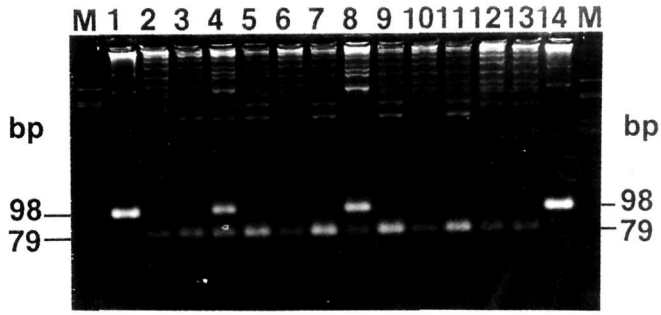

Figure 4. N-*ras* amplification products (98bp) were digested with the restriction endonuclease MspI and electrophoresed through a 4% agarose gel. Lane M: molecular weight marker pUC18/HaeIII, lanes 1,14: positive control undigested PCR product (98bp), lanes 2,3,5,6,7,9,10,11,12,13: negative samples, lanes 4,8: positive samples (79bp).

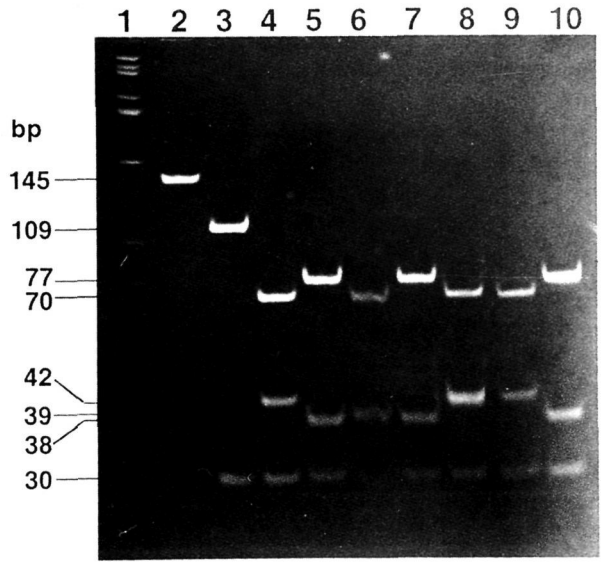

Figure 5. Type distinction of HPV using the general primers. PCR products were digested with the restriction endonuclease RsaI and electrophoresed through a 10% polyacrylamide gel. Lane 1: molecular weight marker pUC18/HaeIII, lane 2: undigested PCR product, lane 3: control HPV11 (109/30bp), lane 4: control HPV16 (30/42/70bp), lane 5: control HPV18 (30/38/77bp), lane 6: control HPV33 (30/39/70bp), lanes 7,10: samples positive for HPV18, lanes 8,9: samples positive for HPV16.

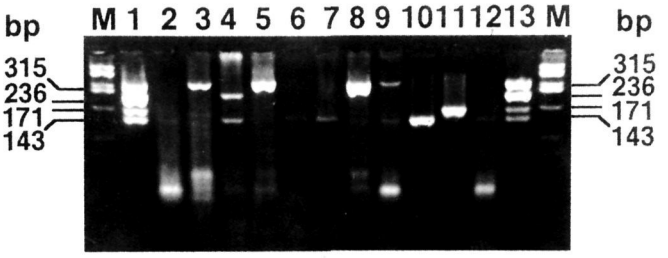

Figure 6. Type distinction of HPV employing a multiplex PCR. Products of different size (315bp: HPV16, 236bp: HPV11, 171bp: HPV33 and 143bp: HPV18) were analysed by agarose gel electrophoresis. Lane M: molecular weight marker pUC18/HaeIII, lane 1,13: control plasmids pHPV16, pHPV11, pHPV33, pHPV18, lane 2,6,7,10,12: samples positive for HPV18, lane 3,9: samples positive for HPV16-HPV18, lane 4: sample positive for HPV11-HPV18, lane 5: sample positive for HPV16, lane 8: sample positive for HPV16, lane 11: sample positive for HPV33.

(6.9%) patients with squamous cell cervical carcinoma (1 case well and 1 case moderately differentiated).

Fifty-eight out of the 112 genital lesions were found positive for HPV (Figs. 5 and 6) with the highest incidence occuring in squamous cell carcinomas of the cervix (79.3%) while in intraepithelial and benign lesions of the cervix, the incidence was lower (70.0% and 40.5% respectively, p=0.001). The results of PCR analysis of the 112 samples are summarized in Table I.

K-*ras* mutations and HPV were more frequently found in malignant lesions of the cervix (28.2% and 76.9% respectively) than in benign (5.4% and 40.5% respectively, p<0.008), while in malignant cervical lesions with K-*ras* mutations, HPV was often detected in the same sample (25.6% vs 2.7%, p=0.004). The highest incidence of K-*ras* mutations was observed in squamous cell carcinomas of the cervix (24.1%), while K-*ras* mutations also occured in cases of cervicitis (5.4%). A comparative analysis between benign and malignant lesions of the cervix is shown in Table II,a. Results from the type distinction of HPV indicated that HPV18 was the most frequent type (with an incidence of 30.2%) while HPV16 was found at a lower rate (27.9%). HPV6 was not detected in any lesion, while HPV11 was more prevalent in benign lesions, often as a co-infection with HPV18 (Table II,b).

Correlation between the presence of *ras* mutations and HPV with the histological differentiation of cervical tumors is summarized in Table III. The frequency of *ras* mutations was related to histological differentiation of the tumor, since it was higher in well differentiated (50%) than in moderately differentiated tumors (8%, p=0.011), although the stage was not available for all the tumors. The rate of HPV DNA detection was more frequent in patients with poorly differentiated (85.7%) than in patients with moderately or well differentiated tumors (44% and 33% respectively, p<0.059).

Discussion

The multi-event nature of carcinogenesis has led to extensive studies of oncogenes, onco-suppressor genes and viral factors involved in animal and human cancers. The collaboration of

Table I. Detection of HPV and mutations of the K-*ras*, H-*ras* and N-*ras* oncogenes in human genital lesions by PCR-RFLP analysis.

Histological diagnosis	Number of patients	HPV positive (%)	Mutations in codon 12 (%) K-*ras*	H-*ras*	N-*ras*
Cervix of the uterus					
Cervicitis	37	15 (40.5)	2	2	1
CIN (I, II, III)	10	7 (70)	1	2	-
Adenocarcinoma	7	5 (71.4)	2	1	-
In situ squamous cell carcinoma	1	-	1	-	1
Squamous cell carcinoma	29	23 (79.3)	7 (24.1)	2 (6.5)	2 (6.9)
Mucoepidermoid carcinoma	1	1	1	-	-
Clear cell carcinoma	1	1	-	-	-
Corpus of the uterus					
Endometrial adenocarcinoma	14	5	1 (7.1)	2 (14.3)	-
Endometrial clear cell carcinoma	1	-	-	-	-
Leiomyosarcoma of the uterus	1	-	-	-	-
Ovary					
Adenocarcinoma	5	-	1	-	-
Borderline mucinous tumor	1	-	-	-	-
Serous cystadenocarcinoma	1	-	-	-	-
Krukenberg tumor	1	-	-	-	-
Vulva					
Squamous cell carcinoma	2	1	-	-	-
Total	112	58	16	9	4

Table II. a. Comparative study of HPV infection and codon 12 of the *ras* gene mutations in cervical lesions.

Types of cervical lesions	Number of patients	HPV positive (%)	K-*ras* mutations (%)	H-*ras* mutations (%)	N-*ras* mutations (%)	With HPV and *ras* gene mutation (%)	Without HPV and *ras* gene mutation (%)
Benign	37	15 (40.5)	2 (5.4)	2 (5.4)	1 (2.7)	1 (2.7)	18 (48.6)
Premalignant	10	7 (70)	1 (10)	2 (20)	0	2 (20)	2 (20)
Malignant	39	30 (76.9)	11 (28.2)	3 (7.7)	3 (7.7)	10 (25.6)	4 (10.3)

Table II. b. Rate of prevalence of HPV type in cervical lesions.

Type of HPV	% HPV positive lesions Benign	Premalignant	Malignant	Total rate
HPV-11	27	30	5	17
HPV-16	19	30	36	27.9
HPV-18	22	30	38.5	30.2
HPV-33	3	10	10	7

ras oncogene with HPV E6/E7 genes in inducing full transformation of cervical keratinocytes has been well documented (13,14). However the exact role of the *ras* oncogene in human carcinogenesis is not yet clear, despite the fact that *ras* mutations occur with variable frequency in a wide variety of tumors (26). Previous studies have shown that the incidence of *ras* mutations in cervical carcinomas is low (11,18).

The frequency found in the current study for K-*ras* mutations was significantly higher in malignant cervical lesions (28.2%) than in benign precursors (5.4%, p=0.008). The frequency of *ras* mutations was higher in well than in moderately differentiated tumors, while no mutations were

Table III. Analysis of the 39 samples with malignant cervical lesions.

Histological differentiation	Total number of cases	Presence of HPV (%)	Presence of *ras* mutation (%)	Presence of *ras* mutation and HPV (%)	Absence of *ras* mutation and HPV (%)
Well differentiated	6	2 (33)	3 (50)[a]	1 (16.7)	0
Moderately differentiated	25	11 (44)	2 (8)	9 (36)	3 (12)
Poorly differentiated	7	6 (85.7)	0	0	1 (14.3)
Undifferentiated	1	1	0	0	0

[a]1 sample carries point mutations in codon 12 of both K-*ras* and H-*ras* genes.

found in poorly differentiated tumors. The relatively high incidence of *ras* mutations in patients with chronic cervicitis, clinically considered as benign lesion, is also of great interest. A future report on the clinical follow-up will help to elucidate its significance as a prognostic factor. The appearance of *ras* mutations in benign and premalignant lesions may contribute to their transition to more advanced lesions. This indicates a possible role of *ras* mutations in the initial stages of cervical carcinogenesis.

In endometrial adenocarcinomas the frequency of all *ras* gene mutations (21.4%) concurred with previous observations (18-20). Although there are reports on codon 12 point mutations of K-*ras*, none exists in H-*ras* gene.

Variations in the prevalence of HPV types in cervical lesions have been found and attributed to geographical differences, focal heterogeneity of HPV replication within lesions sampled, or variability in the sensitivity of the assays employed (24). In areas with high-incidence of cervical cancer there seems to be little difference in the prevalence of HPV16 and HPV18, whereas in areas with moderately-high incidence, HPV16 is more common than HPV18 (27). In the current study we found a higher rate of infection by HPV18 (30.2%) compared to HPV16 (27.9%) in the 86 cervical lesions examined. HPV18 infection was more commonly associated with poorer histological differentiation and our results are substantiated with previous observations that HPV18 confers greater aggression than HPV16 and has been associated with a more rapid progression to malignancy (28). In addition *in vivo* and *in vitro* studies have often indicated that HPV18 is more effective in immortalization and transformation than HPV16 (29).

The simultaneous presence of *ras* mutations and high risk HPV DNA was detected in 10 cases. Its significance is not known, but it has been shown that *in vitro* E6-E7 immortalized primary human epidermal keratinocytes can be rendered malignant after additional transfection with v-*ras* oncogene, while H-*ras* can overcome the anti-proliferative and anti-transformation effects of wild-type p53 in NIH3T3 cells (13-15). Since the simultaneous presence of HPV and K-*ras* mutations was more frequently detected in malignant cervical lesions (25.6%) than in benign (2.7%, p=0.004), a cooperation between them in neoplastic change is suggested.

These experiments support a multistep process for malignant conversion, since for tumors that do not contain any detectable *ras* mutation alternative genetic alterations are presumed. The availability of human epithelial cell transformation model should facilitate studies of the interaction between HPV and human epithelial cells. *ras* activation combined with HPV infection may be an important step in substantial numbers of cervical carcinomas, while their interaction with other genes or events may also be involved.

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