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Detection of HPV and *ras* gene mutations in cervical smears from female genital lesions

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Abstract. The collaboration of *ras* oncogenes with HPV E6/E7 genes inducing full transformation of cervical keratinocytes has been suggested. The purpose of this study was to detect and type HPV by PCR and to assess the extent of involvement of the *ras* oncogene activation by point mutations in cervical premalignant and malignant lesions, using stained PAP cervical smears. Specimens were obtained from 35 women with genital lesions, and codon 12 point mutations of the K-*ras* and H-*ras* oncogenes were detected, as well as HPV18 at a higher rate than HPV16 (48% vs 10%) in cervical lesions by PCR-RFLP and PCR analysis, respectively. Our study indicates that the mutational activation of the K-*ras* gene may be involved in the development of a small subset of cervical carcinomas and that mutationally activated *ras* oncogenes cooperate with HPV in the early stages of carcinogenesis in the cervix of the uterus.

Introduction

Alterations in the cellular genome affecting the expression or function of genes controlling cell growth and differentiation are concidered to be the main cause of cancer. The *ras* family of genes is frequently found to harbor mutations which convert them into active oncogenes (1).

The three forms of Ras: K-*ras*, H-*ras* and N-*ras*, encode for 21 kDa proteins (p21) located in the inner plasma membrane. Ras proteins are membrane bound GTPases. Normal $p21^{ras}$ hydrolyses GTP while mutant $p21^{ras}$ results in the loss of GTPase activity and activation of the gene product (1,2). Normal Ras proteins are involved in the control of cell growth and differentiation but any one of many single amino acid mutations can give rise to highly oncogenic proteins (activating point mutations in *ras* oncogenes occur at codons 12, 13 or 61). The final result of p21 activation is the induction of other genes which stimulate cell proliferation (3). Activated *ras* genes by point mutations have been reported in cervical cancer, although at low frequency (4) while in endometrial carcinomas the frequency was significantly higher (4-6). Mutations of the K-*ras* oncogene have also been reported in human mucinous ovarian tumours (7), in breast (8) and in a wide range of human tumours (1).

Molecular biology techiques have disclosed at least 77 different types and subtypes of HPV (9). An association of HPVs with anogenital lesions can be subdivided into the 'low risk' HPVs such as HPV6 and HPV11 and the 'high risk' HPVs such as HPV16 and HPV18. Specific viral genes (E6 and E7) of high risk HPVs act as oncogenes. High risk E6 and E7 bind and functionally inactivate tumour suppressor proteins p53 and pRb respectively, and both disrupt the G1 arrest in response to DNA damage. 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. Furthermore, it has been demonstrated that the *ras* gene can induce tumorigenic conversion of HPV immortalized cervical keratinocytes (11,12) indicating a cooperative effect between the *ras* and E6/E7 genes in cellular transformation.

In the current study the presence of codon 12 point mutations of K-, H- and N-*ras* genes, as well as detection and identification of the human papillomavirus was examined in 35 cases of genital lesions. Six of the 35 (17%) and 3 of the 35 (8.5%) samples were found to carry a point mutation in K-*ras* and H-*ras* codon 12, respectively. No point mutation of codon 12 of N-*ras* was found. Twenty-nine out of 35 (83%) genital lesions were found positive for HPV and HPV18 was the most frequent type with an incidence of 48%.

Materials and methods

Patients. Specimens were obtained from 35 women with genital lesions, treated at the Department of Cytology, General Hospital of Nikaias, Piraeus. The cervical smears were already fixed and stained to obtain the cytological diagnosis.

DNA extraction. For each case, one slide with stained cervical smear was used. The slides were soaked for 48 h in xylene,

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followed by ethanol washes, to remove the coverslip (13). The cells were scraped into a 1.5 ml Eppendorf tube with 400 µl digestion buffer, containing 150 mM NaCl, 400 mM Tris-HCl, 60 mM EDTA, 15% SDS pH 8.0 and 0.1 mg/ml proteinase K. Samples were then incubated at 60°C for 2 days. Fresh proteinase K was added 3 times daily. The samples were extracted once with phenol/chloroform and once with chloroform. DNA was precipitated with the addition of 20 µl 5 M NaCl and 1 ml ethanol, recovered with centri-fugation at 13,000 rpm for 15 min at 4°C, washed once with cold 70% ethanol and resuspended in 50 µl double distilled water.

Oligonucleotide primers and PCR amplification. All specimens were examined for the presence of amplifiable DNA using a set of primers for, ß-globin gene. The oligonucleotides used for K-*ras* and H-*ras* codon 12 (14) and N-*ras* (15) have been previously described. For the detection and typing of the HPV the general primers GP5 and GP6 (16) and specific primers (17) were used to amplify each virus type (HPV 11, 16, 18 and 33) by multiplex PCR, each virus type giving different length of amplified DNA. 0.5 µl of the extracted DNA of each sample was amplified in a volume of 50 µl containing 200 mM Tris-HCl pH 8.4, 500 mM KCl, 1.5 mM $MgCl_2$, 150-200 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 samples were subjected to 35 cycles of amplification at 94°C for 55 sec, 58°C for 45 sec and 72°C for 45 sec (K-*ras*); 94°C for 55 sec, 54°C for 45 sec and 72°C for 30 sec (N-*ras*); 94°C for 55 sec, 61°C for 45 sec and 72°C for 45 sec (H-*ras*); 94°C for 50 sec, 52°C for 45 sec and 72°C for 45 sec (HPV). PCR products were analyzed on a 2% agarose gel and photographed on a UV light transilluminator.

Multiplex PCR. Amplification was performed at 94°C for 1 min, 55°C for 50 sec and 72°C for 50 sec. Finally, samples were elongated at 72°C for 5 min. To establish type specificity of primer-directed amplification, each set of primers was tested with template plasmid DNA of the four HPV types (11, 16, 18 and 33). PCR products were analyzed on a 4% agarose gel and photographed on a UV light transilluminator.

RFLP analysis K-ras, N-ras. Aliquots (10-40 µl) of the amplification products were digested for 16 h with 30 U BstNI.

H-ras. Aliquots (10-40 µl) of the amplification products were digested for 16 h with 30 U Msp I. RFLP products were analyzed on an 8% polyacrylamide gel and photographed on a UV light transilluminator. The cell lines SW480 for K-*ras* and EJ for H-*ras* were used as positive control.

Results

The presence of amplifiable DNA, using primers for a fragment of ß-globin gene, was confirmed in all of the 35 stained smears examined (data not shown): There were 23 cervical intraepithelial neoplasias (CIN I:9, CIN II:7, CIN III:7), 2 cervical adenocarcinomas and 10 squamous cell cervical carcinomas.

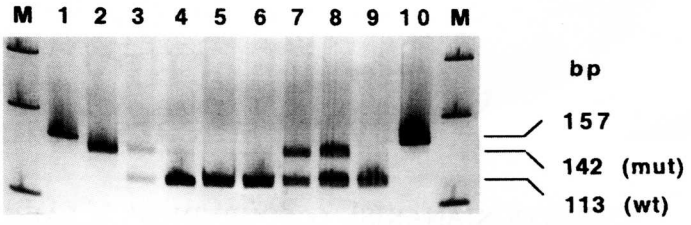

Figure 1. K-*ras* amplification products (157 bp) were digested with the restriction endonuclease BstNI and electrophoresed through an 8% polyacrylamide gel. Lanes 3, 7 and 8, positive samples; lanes 4, 5, 6 and 9, negative samples (113 bp); lanes 2 and 10, positive control SW480 cell line (142 bp); lane 1, undigested PCR product; lane M, 100 bp molecular weight marker.

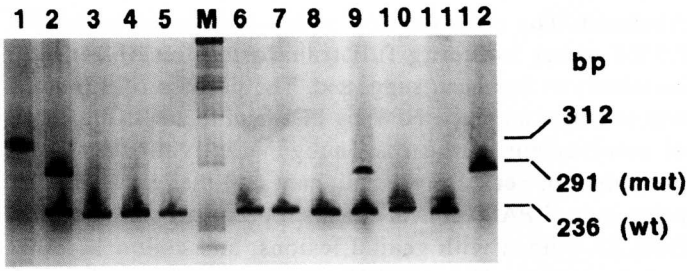

Figure 2. H-*ras* amplification products (312 bp) were digested with the restriction endonuclease MspI and electrophoresed through an 8% polyacrylamide gel. Lanes 2 and 9, positive samples; lanes 3-8, 10 and 11, negative samples (236 bp); lane 12, positive control EJ cell line (291 bp); lane 1, undigested PCR product; lane M, 100 bp molecular weight marker.

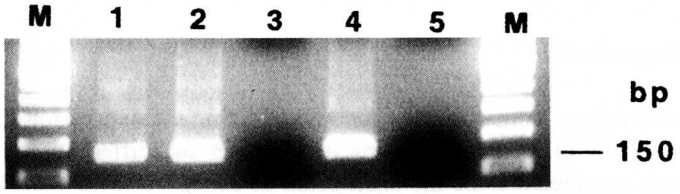

Figure 3. HPV detection using the general primers. PCR products were electrophoresed through a 2% agarose gel. Lanes 1, 2 and 4, positive samples (150 bp); lanes 3 and 5, negative samples; lane M, 100 bp molecular weight marker.

Six out of the 35 (17%) samples were found to carry a point mutation in codon 12 of the K-*ras* gene (for representative examples see Fig. 1). Our study was limited to codon 12 of the K-*ras* gene, since mutations preferentially occur at this codon (18). The K-*ras* mutations were found in 4 out of 23 (17%) patients with cervical intraepithelial neoplasias, in 2 out of 2 (100%) patients with cervical adenocarcinoma and in none of the 10 patients with squamous cell cervical carcinoma. Three out of the 35 (8.5%) samples were found to carry a point mutation in codon 12 of the H-*ras* gene (Fig. 2). H-*ras* mutations were found in 3 out of 7 (8.5%) patients with cervical intraepithelial neoplasia stage III. No sample carried a point mutation in codon 12 of the N-*ras* gene.

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

Histological diagnosis	No. of patients	HPV positive (%)	Mutations in codon 12 (%)		
			K-*ras*	H-*ras*	N-*ras*
Cervix of the uterus					
CIN I, II	16	13 (81)	3 (19)	-	-
CIN III	7	6 (86)	1 (14)	3 (8.5)	-
Adenocarcinoma	2	1 (50)	2 (100)	-	-
Squamous cell carcinoma	10	9 (90)	-	-	-
Total	35	29 (83)	6 (17)	3 (8.5)	-

Table II. HPV typing in human genital lesions by multiplex PCR analysis.

Histological diagnosis	HPV-18	HPV-16	HPV-33	HPV-11	Other types
Cervix of the uterus					
CIN I, II	7	1	-	-	6
CIN III	3	-	1	-	1
Adenocarcinoma	-	-	-	-	1
Squamous cell carcinoma	4	2	-	-	3
Total (%)	14 (48)	3 (10)	1 (4)	-	11 (38)

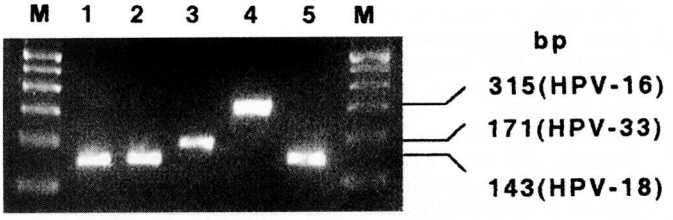

Figure 4. Type distinction of HPV employing a multiplex PCR. PCR products were electrophoresed trough a 4% agarose gel. Lanes 1, 2 and 5, samples positive for HPV18 (143 bp); lane 3, sample positive for HPV33 (171 bp); lane 4, sample positive for HPV16 (315 bp); lane M, 100 bp molecular weight marker.

Twenty-nine out of 35 (83%) genital lesions were found positive for HPV (Figs. 3 and 4) with the highest incidence occuring in squamous cell carcinoma of the cervix (90%) while in intraepithelial neoplasias and adenocarcinomas the incidence was 83% and 50%, respectively. The results of PCR analysis of the 35 samples are summarized in Table I. HPV18 was the most frequent type with an incidence of 48%. HPV16 and HPV33 were observed at 10% and 3.5%, respectively. HPV11 was not detected in any lesion (Table II).

Discussion

The clarification of the precise role of the genes involved in the development of particular tumours may find important clinical application as molecular tumour markers precede the clinical symptoms, as well as in the establishment of novel therapeutic strategies. The collaboration of *ras* oncogene with HPV E6/E7 genes in inducing full transformation of cervical keratinocytes has been documented (11,12). Moreover, the possibility of HPV E6/E7 genes acting as a synergistic factor with *ras* gene activation, or other carcinogens has been suggested (19). Previous studies have shown that the incidence of *ras* mutations in cervical carcinomas is low (4,20).

The present study indicates that point mutations at codon 12 of the K-*ras* gene (17%) occur at a relatively low frequency in cervical premalignant and malignant lesions obtained from the Greek population. These data suggest a potential role of the *ras* gene mutational activation in the development of a subset of cervical carcinomas. K-*ras* point mutations were detected even in patients with premalignant lesions. This indicates a possible role of *ras* mutations in the initial stages of cervical carcinogenesis.

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 (17). In some geographical areas it has been found that HPV16 is more common than HPV18 (21,22). We found a much higher rate of infection by HPV18 (48%) compared to HPV16 (10%). Koffa *et al* (23) found also a higher rate of HPV18 infection in Greek women in lesions of the reproductive tract. HPV18 is considered more tumorigenic than HPV16 and has been associated with a more rapid progression to malignancy (24).

The simultaneous presence of *ras* mutations and high risk HPV DNA was detected in 9 cases. Its exact significance is not known yet but a cooperation between them in neoplastic change is suggested. Malignant conversion is a multistep process, since for tumours with no detectable *ras* mutation alternative genetic alterations are presumed (23). *ras* activation combined with HPV infection may be an important step in a subset of cervical carcinomas, while their interaction with other genes or events may also be involved.

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