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High prevalence of HPV18 in correlation with *ras* gene mutations and clinicopathological parameters in cervical cancer studied from stained cytological smears

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Abstract. The multi-event nature of carcinogenesis has led to extensive studies of oncogenes, onco-suppressor genes and viruses involved in human cancers. The collaboration of *ras* oncogene with HPV E6/E7 genes inducing full transformation of cervical keratinocytes has also been suggested. The purpose of this study was to detect the presence of codon 12 point mutations of *ras* genes, as well as to detect and identify the human papillomaviruses in stained smears of cervical malignancies and to correlate them with the clinicopathological parameters of the Greek patients. Specimens were obtained from 88 women, codon 12 point mutations of the K-*ras* (30%) and H-*ras* (10%) oncogenes, as well as HPV18 were detected at a higher rate than HPV16 (66% vs 7%) in cervical lesions by PCR-RFLP and PCR analysis, respectively. The statistical analysis of the data demonstrates correlation between the presence of K-*ras* mutations and FIGO stage and between FIGO stage and survival of the patients. It is suggested that *ras* activation combined with HPV infection may be an important step in the carcinogenesis of a substantial number of cervical carcinomas.

Introduction

Human papillomaviruses (HPVs) are small double stranded DNA viruses found in a wide variety of proliferative lesions of epithelial origin. Seventy-seven HPV genotypes, each with a distinct tissue tropism, have thus far been identified (1,2). Phylogenetic tree analysis has shown that most HPVs can be classified into specific groups according to known tissue tropism and oncogenic potential (3,4). Most of the HPVs are found in benign proliferations however several types have been discovered in malignant tumours, especially, cancer of the cervix and other anogenital cancers have been linked to specific HPV infections. The role of HPV has been widely studied and current evidence shows that HPV infection and persistance are necessary for the evolution and maintenance of cervical cancer (5). Specific viral genes (E6 and E7) of high risk HPVs (types 16, 18, 33 and others) act as oncogenes. Their expression appears enhanched in premalignant and malignant genital tumours and emerges as a necessary but not a sufficient factor for malignant conversion. These viral oncogenes are responsible for the genetic instability of the infected cells. High risk E6 and E7 bind and functionally inactivate tumour suppressor proteins p53 and Rb respectively, and both disrupt the G1 arrest in response to DNA damage.

As in the case for other DNA tumour 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 (6), 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 (7,8). Furthermore, it has been demonstrated that the *ras* gene can induce tumorigenic conversion of HPV-immortalized cervical keratinocytes (9,10) indicating a cooperative effect between *ras* and E6/E7 genes in cellular transformation.

The *ras* family of genes is frequently found to harbour mutations which convert them into active oncogenes. The three forms of *ras*: K-*ras*, H-*ras* and N-*ras* encode for 21 kDa protein (p21) located in the inner plasma membrane. Ras proteins are membrane bound GTPases. The commonest mechanism for the activation of the *ras* family genes are point mutations in codons 12, 13 and 61 which abolish p21 GTPase activity and thus, remain constitutively activated (11). Activated *ras* genes by point mutations have been reported in cervical cancer, although at low frequency (12) while in endometrial carcinomas the frequency was

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significantly higher (12-14). K-*ras* is the member of the *ras* family which harbours point mutations in the highest frequency. Mutations of the K-*ras* oncogene have also been reported in human mucinous ovarian tumours (15), in breast (16) and in a wide range of human tumours (17).

In the current study, in an effort to extend our initial findings (18), we examined the presence of codon 12 point mutations of K-, H- and N-*ras* genes, as well as detection and identification of the human papillomaviruses in 88 cases of cervical lesions. Twenty-six of the 88 (29.5%) and 9 of the 88 (10%) samples were found to carry a point mutation in K-*ras* and H-*ras* codon 12 respectively. No point mutation of codon 12 of the N-*ras* was found. Fifty-five out of 88 (62.5%) cervical lesions were positive for HPV, HPV18 being the most frequent type with an incidence of 65.5%.

Materials and methods

Patients. The study was based on 88 cytologic smears of cervical lesions stained with the Papanikolaou method. The cervical smears were already fixed and stained to obtain the cytological diagnosis. The specimens were obtained from the pathology archives for the period 1989-1995 from the Department of Cytology, Alexandra Hospital, Athens. Specimen assessment was also confirmed histologically.

DNA extraction. For each case, one slide with stained cervical smear was used. The slides were soaked for 48 h in xylene, followed by ethanol washes, to remove the coverslip. 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 centrifugation at 13000 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 and N-*ras* codon 12 (20) have been previously described. For the detection and distinction of the HPV general primers GP5 and GP6 (21) and specific primers (22) were used to amplify each virus type (HPV 11, 16, 18 and 33) by multiplex PCR, each virus type giving different lengths of amplified DNA. Specimens positive for HPV18 using the first set of primers (22) were also confirmed applying another set of primers: HPV18-1: 5' tgcaaccgaaataggttgggc 3' and HPV18-2: 5' gcacagatcaggtagcttgta 3'. The extracted DNA (0.5 µl) 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. In each PCR reaction two blank samples were employed as negative controls to ensure that no contaminants were introduced. 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 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 3% agarose gel and photographed on a UV light transilluminator. The mixture containing the HPV18-1,2 set of primers was heated for 1 min at 95°C and samples were subjected to 35 cycles of amplification at 94°C for 40 sec, 61°C for 40 sec and 72°C for 50 sec. Finally, samples were elongated at 72°C for 5 min. PCR products were analyzed on a 2% agarose gel and photographed on a UV light transilluminator.

RFLP analysis. K-*ras*, N-*ras*: 10-40 µl aliquots of the amplification products were digested for 16 h with 30 U BstNI. H-*ras*: 10-40 µl aliquots of the amplification products were digested for 16 h with 30 U MspI. RFLP products were analyzed on an 8% polyacrylamide gel and photographed on a light transilluminator. The cell lines SW480 for K-*ras* and EJ for H-*ras* were used as positive control.

Statistical analysis. The presence of K-*ras* and H-*ras* was analyzed for correlation with the type of HPV, histological type and grade, FIGO stage, and survival of patients from a follow-up study. Statistical analysis of the results was performed with the package SPSS 6.0 (for Windows). Statistical significance was set at $p \leq 0.05$.

Results

Clinicopathological data and patient course. Cases were classified according to the Histological Typing of Female Genital Tract Tumors by the World Health Organization (23) as follows: there were 11 cervical intraepithelial neoplasias (CIN III: 11), 2 cervical adenosquamous carcinomas, 75 squamous cell cervical carcinomas of the cervix.

The FIGO stage (24) was known for 44 (50%) patients: 8 (18%) were stage I, 21 (48%) stage II, 12 (27%) stage III and 3 (7%) stage IV. Tumours were also classified according to their degree of histological differentiation (25): 19% grade I (G1), 67% grade II (G2) and 14% grade III.

Follow-up was completed for 50% of the patients. Fifty-two per cent have died, 2% died of unrelated cause and 46% have no evidence of recurrence (4-10 years after surgery).

Mutation frequency of ras at codon 12. The presence of amplifiable DNA, using primers for a fragment of ß-globin gene, was confirmed in all the stained smears examined (data not shown).

Twenty-six out of the 88 (39%) 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

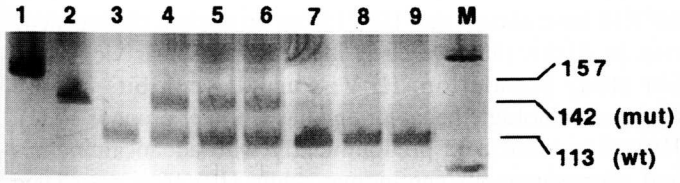

Figure 1. K-*ras* amplification products (157 bp) were digested with the restriction endonuclease BstNI and electrophoresed through an 8% polyacrylamide gel. Lanes 4-6, positive samples; lanes 3,7-9, negative samples (113 bp); lane 2, positive control SW480 cell line (142 bp); lane 1, undigested PCR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

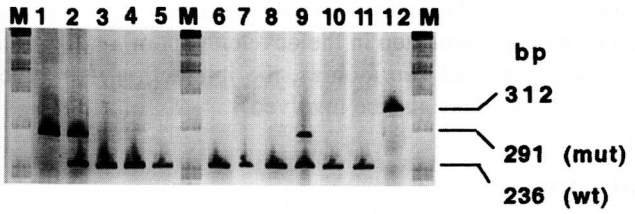

Figure 2. H-*ras* amplification products (312 bp) were digested with the restriction endonuclease MspI and electrophoresed through an 8% polyacrylamide gel. Lanes 2 and 9, positive samples; lanes 3-8,10,11, negative samples (236 bp); lane 1, positive control EJ cell line (291 bp); lane 12, undigested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

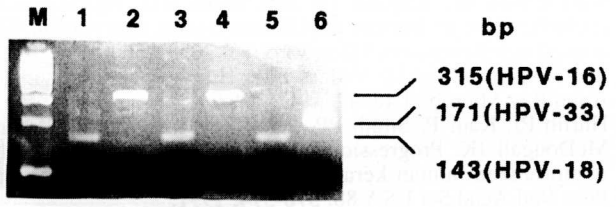

Figure 3. Type distinction of HPV employing a multiplex PCR. PCR products were electrophoresed trough a 3% agarose gel. Lanes 1, 3 and 5, samples positive for HPV18 (143 bp); lane 6, sample positive for HPV33 (171 bp); lanes 2 and 4, samples positive for HPV16 (315 bp); lane M, 100 bp molecular weight marker.

codon 12 of the K-*ras* gene, since mutations preferentially occur at this codon (26). The K-*ras* mutations were found in 2 out of 11 (18%) patients with cervical intraepithelial neoplasias, in 24 out of 75 (32%) patients with squamous cell cervical carcinomas of the cervix. Nine out of the 88 (10%) samples were found to carry a point mutation in codon 12 of the H-*ras* gene (Fig. 2). No sample with a point mutation in codon 12 of the N-*ras* gene was found. H-*ras* mutations were detected in 2 out of 11 (18%) patients with cervical intraepithelial neoplasias and in 7 out of 72 (10%) patients with squamous cell cervical carcinomas.

Presence and type of HPV. Fifty-five out of 88 (63%) genital lesions were found positive for HPV (Fig. 3) with the highest incidence occurring in squamous cell cervical carcinomas (78%). The results of PCR analysis and the histology of the samples are summarized in Table I.

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 III	11	10	2	2	-
Adenosq ca	2	1	-	-	-
Sq cell ca	75	44	24	7	-
Total	88	55 (63)	26 (30)	9 (10)	-

ca, carcinoma; sq, squamous.

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

Histological diagnosis	HPV18	HPV16	HPV33	HPV11	Other types
Cervix of the uterus					
CIN III	8	2	-	-	-
Adenosq ca	-	-	-	-	1
Sq cell ca	28	2	3	-	11
Total (%)	36 (66)	4 (7)	3 (5.5)	-	12 (22)

Ca, carcinoma; sq, squamous.

HPV18 was the most frequent type with an incidence of 66%. Presence of HPV18 using primers HPV18-1,2 was confirmed in all the positive cases found (data not shown). PV16 and HPV33 were found at lower rates (7 and 5.5% respectively) (Table II). No sample had the HPV11 type.

Relationship of clinicopathological features. Clinical and pathological details of the patients with mutations at codon 12 of the K-*ras* and H-*ras,* and the HPV type are reported in Table III.

The statistical analysis of the data demonstrate correlation between the presence of K-*ras* mutations and FIGO stage (p=0.039). Also, there was a correlation between FIGO stage and survival of the patients (p=0.049).

Statistical correlation was observed between HPV type and survival of the patients (p=0.107) and the presence of K-*ras* and the clinical outcome of the patients (p=0.182).

Discussion

Mutations in *ras* oncogenes occur in a wide variety of tumours but the precise role of *ras* oncogenes in carcinogenesis is not

Table III. Analysis of K-*ras* and H-*ras* codon 12 mutations, type of HPV in cervical lesions according to the clinico-pathological parameters of the patients.

Parameter	No. of patients	K-*ras*	H-*ras*	HPV type		
				HPV18	HPV33	Other
Tumour stage						
1 (G1)	8	4	3	-	1	1
2 (G2)	29	12	4	9	-	4
3 (G3)	7	1	-	1	-	1
FIGO stage						
I	8	2	-	2	-	2
II	21	2	3	1	1	3
III	12	-	2	9	1	3
IV	3	-	-	1	-	-
Fate						
Alive	20	4	2	3	2	6
Dead	23	2	1	11	1	3
Dead[a]	1	1	-	1	-	-

[a]Dead, died of an unrelated cause.

yet clear. Moreover, the possibility of HPV E6/E7 genes acting as a synergistic factor with *ras* gene activation, or other carcinogens has been suggested.

The current study evaluated mostly specimens derived from malignant cervical lesions, and it suggests that point mutation at codon 12 of the K-*ras* gene (30%) may have a potential role in the development and/or the progression of the multistep process of cervical carcinogenesis. K-*ras* and H-*ras* mutations were detected not only in malignant lesions but also in patients with carcinoma *in situ*. Other studies (26) have focused on premalignant and malignant samples indicating a possible role of *ras* mutations in the initial stages of cervical carcinogenesis.

In our study, statistical correlation was found between K-*ras* point mutations and FIGO stage indicating that the presence of K-*ras* mutations coexists with the increase of the FIGO stage. Moreover, some degree of correlation was found between the presence of K-*ras* gene mutations and the survival of the patients.

Variations in the prevalence of the 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 (27). In some geographical areas it has been found that HPV16 is more common than HPV18 (28). We found a much higher rate of infection by HPV18 (66%) compared to HPV16 (7%). Other studies performed in Greek women with cervical lesions confirm this finding (18,29). A possible explanation for the overepresentation of HPV in the Greek population could be attributed to ethnic variations of HPV types. Previous studies regarding the molecular evolution of HPV18 have shown that HPV18 has an ancient phylogenetic root in Africa (30), which is located in the near vicinity of our study population. HPV18 usually exhibits a more aggressive biologic behavior and has a poorer prognosis (31). HPV18 is associated predominantly with adenocarcinomas and adenosquamous carcinomas while HPV16 is associated predominantly with squamous cell carcinomas which have better prognosis.

In the present study, statistical correlation was also found between these specific HPV types and the clinical outcome of the patients. The simultaneous presence of *ras* mutations and high risk HPV DNA was detected in 18 cases, suggesting a possible cooperation between them in neoplastic change.

In conclusion, *ras* activation combined with HPV infection may be an important step in the development of a substantial number of cervical carcinomas, the interaction with other genes or events may also be involved.

References

1. De Villiers E-M: Heterogeneity of the human papillomavirus group. J Virol 63: 4898-4903, 1989.
2. De Villiers E-M: Human pathogenic papillomavirus types. In: Human Pathogenic Papillomaviruses. Zur Hausen H (ed). Springer-Verlag, Berlin, pp1-12, 1994.
3. Chan SY, Bernard HU, Ong CK, Chan SP, Hofmann B and Delius H: Phylogenetic analysis of 48 papillomavirus types and 28 subtypes and variants: a showcase for the molecular evolution of DNA viruses. J Virol 66: 5714-5725, 1992.
4. Van Ranst M, Kaplan JB and Burk RD: Phylogenetic classification of human papillomaviruses: correlation with clinical manifestations. J Gen Virol 73: 2653-2660, 1992.
5. Zur Hausen H and de Villers EM: Human papillomaviruses. Annu Rev Microbiol 48: 427-447, 1994.
6. Hurlin PJ, Kaur P, Smith PP, Perez-Reyes N, Blanton RA and McDougall JK: Progression of human papillomavirus type 18-immortalized human keratinocytes to a malignant phenotype. Proc Natl Acad Sci USA 88: 570-574, 1991.
7. Riou G, Barrois M, Shenz Z-M, Duvillard P and Lhomme C: Somatic deletions and mutations of c-Ha-*ras* gene in human cervical cancers. Oncogene 3: 329-333, 1988.
8. Riou G, Le MG, Favre M, Jeannel D, Bourhis J and Orth G: Human papillomavirus-negative status and c-*myc* gene over-expression: independent prognostic indicators of distant metastasis for early-stage invasive cervical cancers. J Natl Cancer Inst 84: 1525-1526, 1992.
9. DiPaolo JA, Woodworth CD, Popescu NC, Notario V and Doniger J: Induction of human cervical squamous cell carcinoma by sequential transfection with human papillomavirus 16 DNA and viral Harvey *ras*. Oncogene 4: 395-399, 1989.
10. Dürst M, Gallahan D, Gilbert J and Rhim J-S: Glycocorticoid enhanced neoplastic transformation of human keratinocytes by human papillomavirus type 16 and an activated *ras* oncogene. Virology 173: 767-771, 1989.
11. Pronk GJ and Bos JL: The role of p21 *ras* in receptor tyrosine kinase signalling. Biochem Biophys Acta 1198: 131-147, 1994.
12. Enomoto T, Inoue M, Perantoni AO, Buzard GS, Miki H and Tanizawa O: K-*ras* activation in premalignant and malignant epithelial lesions of the human uterus. Cancer Res 51: 5308-5314, 1991.
13. Trowbridge DI, Risinger JI, Dent GA, Kohler M, Berchuck A, McLachlan JA and Boyd J: Mutations of the Ki-*ras* oncogene in endometrial carcinoma. Am J Obstet Gynecol 167: 227-232, 1992.
14. Mizuuchi H, Nasim S, Kudo R, Silverberg SG, Greenhouse S and Garrett CT: Clinical implications of K-*ras* mutations in malignant epithelial tumors of the endometrium. Cancer Res 52: 2777-2781, 1992.
15. Ichikawa Y, Nishida M, Suzuki H, Yoshida S, Tsunoda H, Kubo T, Uchida K and Miwa M: Mutation of K-*ras* proto-oncogene is associated with histological subtypes in human mucinous ovarian tumors. Cancer Res 54: 33-35, 1994.