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 persistence 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 enhanced 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
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 β-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’ gcaacaggaatagggtggtc 3’ and HPV18-2: 5’ ggacagacgagtagtgtga 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 MgCl2, 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 Mspl. 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≤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 β-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
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.

<table>
<thead>
<tr>
<th>Histological diagnosis</th>
<th>No. of patients</th>
<th>HPV positive (%)</th>
<th>Mutations in codon 12 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>K-ras</td>
<td>H-ras</td>
</tr>
<tr>
<td>Cervix of the uterus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN III</td>
<td>11</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Adenosq ca</td>
<td>2</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Sq cell ca</td>
<td>75</td>
<td>44</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>88</td>
<td>55 (63)</td>
<td>26 (30)</td>
</tr>
</tbody>
</table>

ca, carcinoma; sq, squamous.

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

<table>
<thead>
<tr>
<th>Histological diagnosis</th>
<th>HPV18</th>
<th>HPV16</th>
<th>HPV33</th>
<th>HPV11</th>
<th>Other types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervix of the uterus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN III</td>
<td>8</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adenosq ca</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Sq cell ca</td>
<td>28</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>Total (%)</td>
<td>36</td>
<td>4</td>
<td>3</td>
<td>5.5</td>
<td>12 (22)</td>
</tr>
</tbody>
</table>

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

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 through 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 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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of patients</th>
<th>K-ras</th>
<th>H-ras</th>
<th>HPV type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumour stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (G1)</td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>2 (G2)</td>
<td>29</td>
<td>12</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>3 (G3)</td>
<td>7</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>8</td>
<td>2</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>21</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>III</td>
<td>12</td>
<td>2</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>IV</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>20</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Dead</td>
<td>23</td>
<td>2</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Dead*</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

*Dead, died of an unrelated cause.

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


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 overrepresentation of HPV in the Greek population could be attributed to ethnic variations of HPV types. Previous studies regarding the molecular evolution of


