High-Risk Human Papillomavirus Infections and Overexpression of p53 Protein as Prognostic Indicators in Transitional Cell Carcinoma of the Urinary Bladder

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ABSTRACT

Ninety Japanese patients with transitional cell carcinoma of the urinary bladder were investigated for tumor incorporation of DNA for high-risk human papillomavirus (HPV) types 16, 18, and 33 by in situ hybridization with biotinylated DNA probes. In addition, immunohistochemical analysis of p53 protein expression was performed with an antibody to p53 protein. Twenty-eight tumors were positive for HPV DNA, and multiple HPV infection was detected in 17 cases. Positive nuclear staining of cancer cells by the antibody to p53 protein was detected in 33 cases. DNA for HPV 16, 18, and/or 33 and the overexpression of p53 protein were simultaneously observed in 6 tumors by using a mirror section method. The overexpression of p53 protein was frequently detected in invasive and nonpapillary tumors (P < 0.05) and in high grade tumors (P < 0.05). In contrast, HPV infection was more common in noninvasive and papillary tumors (P < 0.01). The patients with tumors positive for HPV DNA and/or p53 antibody had a significantly worse survival rate (P < 0.05). These results suggest that HPV infection or overexpression of p53 protein may be related to tumor behavior and may indicate a relatively poor prognosis in patients with transitional cell carcinoma.

INTRODUCTION

Infection with high-risk HPV types 16, 18, and 33 has been detected in a high percentage of patients with several types of cancer, suggesting that it may be a risk factor for carcinoma of the uterine cervix (1), anus (2), and esophagus (3-5). On the other hand, HPV infection has also been confirmed in TCC of the urinary bladder (6-8). Anwar et al. (8) investigated the prevalence of HPV on neoplastic or normal urinary bladder specimens and revealed the increased infection of high-risk HPV in tumors as compared with nonneoplastic tissues.

The oncogene products of several DNA tumor viruses appear to target the retinoblastoma gene product and p53 proteins. For example, the E6 and E7 oncoproteins encoded by HPV bind to p53 protein (9) and to the retinoblastoma gene product (10), respectively. Although binding of SV40 T-antigen and adenovirus E1B protein leads to the stabilization of p53, the binding of E6 protein encoded by HPV types 16 and 18 results in rapid degradation of p53 via the ubiquitin-directed pathway (11). Thus, it is clear that the role of the E6 oncoprotein is to eliminate or inactivate p53 as a tumor suppressor, although the functional consequences of p53 binding and degradation by E6 in HPV-induced cancer remain unclear. This hypothesis is supported by the observation that cell lines derived from cervical carcinomas bearing the HPV type 16 or 18 E6 gene product also possess a wild-type p53 gene (12). In fact, no mutant p53 gene or protein has been detected in tumors with the HPV E6 gene, such as cervical carcinomas (12, 13) and esophageal carcinomas (4, 5).

Identification of p53 protein overexpression by immunohistochemical staining has been reported in many kinds of tumors in relation to p53 gene mutation. Overexpression of p53 protein or mutation of the p53 gene has also been demonstrated in a high percentage of bladder carcinomas (14-18). In addition, recent studies have shown that the incidence of p53 gene mutation is high in invasive and high-grade bladder carcinomas (16-18).

In the present study, human TCCs of the urinary bladder were screened for the presence of the DNA for HPV types 16, 18, and 33 for p53 protein expression. From these data, the relationship with various clinicopathological factors and the prognosis were determined.

MATERIALS AND METHODS

Patients and Tumor Samples. A total of 90 human TCCs of the urinary bladder obtained by radical or partial cystectomy between 1981 and 1992 at the Department of Urology of Kochi Medical School and the Division of Urology of Kochi Takasu Hospital were studied. The patients with TCC included 69 men and 21 women, ages 49-92 years. Histological or clinical classification was performed according to "The General Rules for Clinical and Pathological Studies on Bladder Cancer of the Japanese Urological Association and the Japanese Pathological Society." All tumor specimens were fixed in 10% buffered formalin, processed routinely, and embedded in paraffin. In each case, all the available hematoxylin and eosin-stained sections were reviewed, and a representative block was chosen for further studies.

In Situ Hybridization and Immunoperoxidase Staining. In situ hybridization was performed with biotinylated probes specific for the DNA of HPV types 16, 18, and 33 (DAKO Japan, Kyoto, Japan), and p53 protein expression was assessed by immunohistochemical examination with a monoclonal antibody (DAKO-p53 Protein; DO-7; dilution, 1:30) as reported previously (5). For immunohistochemical staining, deparaffinized tissue sections were placed in deionized water and heated to 95 ± 5°C in a microwave oven for 5 min (19).

In situ hybridization with each HPV DNA probe was performed using an in situ hybridization kit (DAKO). Immunohistochemical staining for p53 was done by the avidin-biotin complex procedure using a streptavidin-biotin complex peroxidase kit (Histofine SAB-PO kit; Nichirei, Inc., Tokyo, Japan). The specificity of the DNA probes and antibody used in this study was checked by using positive or negative control sections of various kinds of tissues.

Mirror Section Analysis of HPV DNA and p53 Positivity. Two serial 3-μm-thick sections were obtained with the cut surfaces facing each other. Each section was then individually reacted with an HPV DNA probe (type 16, 18, or 33) by in situ hybridization or with the anti-p53 antibody by immunohistochemistry, as described above.

Statistical Analysis. Statistical associations between HPV infection or overexpression of p53 protein and various clinical or pathological factors were determined using the χ2 test (P < 0.05). Analysis of survival was performed using survival curves and the Kaplan-Meier method. In addition, the Cox proportional hazards model was used to calculate and estimate the postoperative survival rate and to determine the statistical difference for each prognostic factor of histological or clinical classification. For multivariate analysis, variables were selected on the condition that they were statistically significant and only showed a poor correlation with each other (correlation coefficient; P < 0.4).

RESULTS

In Situ Hybridization with HPV DNA Probes and p53 Immunohistochemistry. Overall, 31.1% (28 of 90) of the surgically resected tumors had numerous cancer cell nuclei positive for HPV DNA types 16, 18, and/or 33 (Fig. 1a). The distribution of cancer cells

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3 The abbreviations used are: HPV, human papillomavirus; TCC, transitional cell carcinoma; PN, papillary and noninvasive; NI, nonpapillary and invasive.

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positive for each HPV type was similar, although the proportion of positive cells varied between tumors. Positive staining with the anti-p53 antibody was detected in 35.6% (32 of 90) of the tumors. The staining pattern was a strong reaction restricted to the nucleus in most of the cancer cells (Fig. 1b). Staining was negative in most noncancerous tissue from the patients, including bladder epithelium, uninvolved stroma adjacent to the carcinomas, and invading inflammatory cells. The distribution of each HPV type associated with p53 positivity is shown in Table 1. Six patients were simultaneously positive for HPV types 16, 18, and/or 33, and p53 antibody. The mirror section technique was performed in these 6 cases to identify cancer cell nuclei positive for both HPV DNA and p53 (Fig. 2). In the remaining 36 tumors, neither the detection of HPV DNA nor p53 protein was found.

Statistical Analysis. The tumors were divided into four groups on the basis of the pattern of tumor cell positivity for HPV DNA and/or p53. Group I consisted of 22 tumors (24.4%) that were positive for HPV DNA and negative for p53, whereas Group II contained 26 tumors (28.9%) positive for p53 and negative for HPV. Group III contained 6 tumors (6.7%) showing positive for both HPV DNA and p53, while Group IV consisted of 36 tumors (40.0%) negative for both HPV and p53. Table 2 shows the relationships between the detection of HPV DNA or the overexpression of p53 protein and the various clinical or pathological factors. Regarding the tumor growth patterns, HPV infection (Group I tumors) was associated with PN tumor growth (Fig. 1a) and was statistically significant (P < 0.01). There was only a rare association between HPV infection and tumors with non-papillary and non-invasive growth, or invasive tumors of the papillary and invasive, or NI tumor growth types. The association between p53 protein expression (Group II tumors) and NI tumors (P < 0.05) or lesions with a high nuclear grade (P < 0.05) was also statistically significant (Fig. 1b). In contrast, no significant association was detected between HPV infection or p53 overexpression and other clinical or pathological parameters (patient's sex, age, tumor grade, depth of penetration, lymphatic or venous invasion, distant organ and/or lymph node metastasis, and treatment with radiation therapy or chemotherapy). There was also no significant association between Group III or Group IV tumors and any of the clinicopathological factors.

Association between High-Risk HPV or p53 Expression and Prognosis. The postoperative 10-year survival rate was 45.4% (n = 22) in Group I, 38.8% (n = 26) in Group II, and 71.1% (n = 36) in Group IV. As shown in Fig. 3, there was a significant difference in 5-year (P < 0.05) and 10-year (P < 0.05) survival between Group II and Group IV. Although the curves of Groups I and IV diverged with a worse survival for Group I, no statistical difference in survival was found. In addition, no significant difference in survival was detected between Groups I and II. In Group III, the duration of follow-up ranged from 3 to 57 months and the 57-month (4.8-year) survival rate was 33.3% (n = 6). There was a significant difference between Group III and Group IV in the 57-month survival rate (P < 0.05).

Regarding survival rate, a multivariate analysis using Cox's stepwise proportional hazards model was used to calculate tumor grade, depth of penetration, lymphatic or venous invasion, distant organ and/or lymph node metastasis, tumor growth pattern, and HPV infection and/or p53 overexpression, which were each statistically significant. A stepwise selection of these factors was made based on the relative magnitude of their contribution to survival. Analysis demonstrated that the most important factor affecting survival was the distant organ and/or lymph node metastasis (P < 0.01). HPV infection and/or p53 overexpression was another important factor affecting survival (P < 0.05).

DISCUSSION

We investigated HPV types 16, 18, and 33 infection and the overexpression of p53 protein in 90 patients with TCC of the urinary bladder and assessed the relationship to various clinicopathological factors.
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Factors or prognosis. We used *in situ* hybridization to detect HPV DNA and found it in 31.1% of the tumors, whereas Anwar et al. (8) previously detected HPV DNA in 62% of urinary bladder tumors by gene amplification using polymerase chain reaction. This high incidence of HPV infection almost certainly resulted from the greater sensitivity of polymerase chain reaction when compared with *in situ* hybridization. The mutation of *p53* has recently been identified in many kinds of tumors. We also detected the overexpression of *p53* protein in 35.6% of TCCs and 6 of the tumors showed simultaneous positivity for HPV DNA. To our knowledge, the simultaneous detection of HPV type 16, 18, or 33 and *p53* protein overexpression has not been demonstrated previously in single cancer cell nuclei of bladder TCC. This finding suggests that both HPV infection and overexpression of *p53* protein coexist in some bladder TCCs.

Table 2 Relationship between the high-risk HPV infection and/or overexpression of p53 protein and clinicopathological factors

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<th>Group I: HPV (%)</th>
<th>Group II: p53 (%)</th>
<th>Group III: HPV and p53 (%)</th>
<th>Group VI: others (%)</th>
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<th><strong>Female</strong></th>
<th><strong>Total (%)</strong></th>
<th><strong>Sex</strong></th>
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<td>16 (73)</td>
<td>18 (78)</td>
<td>5 (21)</td>
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*Mult.*, multiple infection of high-risk HPV; pT, depth of penetration; ly or v, lymphatic or venous invasion; m or n, distant organ and/or lymph node metastasis; NN, nonpapillary and noninvasive; PI, papillary and invasive; T. growth, tumor growth pattern; Chem, chemotherapy; Rad, radiation therapy.
Bladder cancer usually exhibits significantly different behavior, depending on the nuclear grade of the cancer cells and the patterns of growth. The present study showed that HPV infection was frequently associated with PN tumors and rarely with other patterns of tumor growth. So-called "superficial" bladder cancers, including PN tumors, usually develop as multiple- and low-grade lesions with a specific papillary shape and frequently recur after transurethral resection. Thus, our results suggest that HPV infection may also be associated with the clinical behavior of superficial bladder cancer. An increase of p53 protein was detected in a high proportion of NI tumors. Recent reports have indicated that point mutations of the p53 gene are closely associated with invasive and high-grade bladder cancer but rarely with superficial and low-grade tumors (16-18). The p53 protein normally has too short a half-life for its accumulation to be detected by current immunohistochemical techniques (20), whereas in tumors the concentration of this protein increased, probably due to increased synthesis and lowered degradation (20, 21). Thus, p53 protein became detectable immunohistochemically (22-25). In addition, the overexpressed p53 protein closely correlated with mutations, allowing an immunohistochemical evaluation of the role of p53 mutations in various tumors (21, 26-28). However, some p53 mutations found in human and animal tumors result in a lack of expression of p53 mRNA and protein (29-31). Recent analysis also suggested that the relationship between p53 mutations and protein expression was not clear-cut and that mutations did not directly reflect p53 overexpression as assessed by immunohistochemistry (20, 21, 27, 28). Bodner et al. (32) also reported that the undetectable level of p53 protein expression was immunohistochemically found in human lung carcinoma cell lines associated with p53 mutations. Although we could not accurately conclude whether the anti-p53 antibody used in this study would react with all mutant forms of the p53 protein, our data support the concept that overexpression of p53 may be an important factor for carcinogenesis.

In a previous study, Furihata et al. (5) investigated the presence and distribution of HPV infection or aberrant expression of p53 protein in human esophageal cancer and revealed that HPV types 16 and 18 infection, and more particularly a high level of p53 expression, may be independent markers of a worse prognosis. Our findings also indicated that the high level of p53 expression in Group II was associated with a significantly poorer prognosis ($P < 0.05$) than that of Group IV. The patients with HPV infection in Group I also had a poorer prognosis than Group IV, while there was no significant difference between Groups I and II. In general, the virally encoded oncoproteins E6 and E7 form a complex with the cell-encoded protein products of tumor suppressor genes. The E6 protein encoded by HPV-16 or -18 binds to cellular p53 protein to promote rapid degradation of this tumor suppressor gene product (11). Several lines of evidence have already suggested that functional inactivation of p53 is important in the immortalization of human epithelial cells and the progression of HPV-infected tumors (11). Recent investigations in various tumors have also shown that the loss of wild-type p53 function is an event of critical importance in oncogenesis (30). Thus, our data may imply that overexpression of p53 protein or high-risk HPV infection contributes to loss of wild-type p53 function. In the present study, Group III showed a poorer prognosis than the other groups. One reason for this may be that both overexpressed p53 protein and HPV may cooperate to promote competition against the functioning of wild-type p53.

In conclusion, our findings indicate that infection with HPV and/or overexpression of p53 protein may be important prognostic indicators and useful for assessing tumor aggressiveness. Further investigations are required in a larger number of TCCs at the DNA or RNA level in order to confirm these results.

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REFERENCES

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