Presence of ras Oncogene Mutations and Human Papillomavirus DNA in Human Prostate Carcinomas

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ABSTRACT

The frequency of H-ras, K-ras, and N-ras mutations and the presence of high-risk human papillomavirus (HPV 16, 18, and 33) DNA were studied in 75 paraffin-embedded specimens obtained from 68 Japanese patients with a variety of prostate carcinomas by using polymerase chain reaction and DNA hybridization with sequence-specific oligonucleotides. Ten specimens each of normal and benign hyperplastic prostatic tissues from the same number of patients were also examined for this analysis. Of 68 carcinoma cases, ras gene mutations were present in 16 cases (24%) and HPV DNAs in 28 cases (41%). Eleven mutations were detected in codon 61 of H-ras, 4 in codon 12 of N-ras, and 2 in codon 61 of K-ras. HPV 16, 18, and 33 DNAs were found in 11, 17, and 5 cases, respectively. Eight of the 16 cases with ras mutation also harbored HPV DNAs. The frequency of ras mutations and the HPV infection increased in patients with advanced stages of the tumor and with the higher Gleason score. There was the predominant presence of H-ras codon 61.2 (CAG—CTG) mutation and HPV 18 DNA in prostatic carcinomas metastasizing to the bone. None of the normal or benign hyperplastic prostatic specimens contained either ras mutation or HPV DNA. Our results suggest that ras gene mutations and HPV infections are relatively frequent, at least in prostate carcinoma of Japanese patients. These two factors appear to be related to the progression of the tumor. Moreover, H-ras codon 61.2 mutation and HPV 18 infection may have some predictive roles for bone metastasis in prostate carcinoma.

INTRODUCTION

Prostatic carcinoma is the second most common cause of cancer in men (1). There are, however, wide geographic and racial differences in the incidence of clinically diagnosed prostatic cancer, ranging from 5.1—8.8 cases/100,000 population in Japan to 41.7—91.2 cases/100,000 in the United States (2). The biological behavior of clinical prostate carcinoma is different in different countries: that in Japanese patients is far more aggressive than that in the Americans (3, 4). It has been suggested that the combination of race and diet can predict some, if not all, of the epidemiological spectrum of prostate cancer.

The recent molecular studies have indicated the important role of protooncogenes and tumor suppressor genes in human carcinogenesis. There is, however, relatively little information about the molecular events in the pathogenesis of prostate carcinoma. Previous works have shown the increase of Ras-p21 protein expression and of c-myc transcript in prostatic carcinoma as compared with normal prostate gland or benign prostatic hyperplasia (1). However, two recent studies (5, 6) from the United States could detect ras mutation only in less than 5% of prostatic cancers analyzed, although the number of cases examined was limited in both reports.

Another factor in carcinogenesis is viral infection. The possible etiological role of high-risk HPV (e.g., HPV 16, 18, and 33) has been suggested, especially in anogenital cancer (7). Recently, 33% of prostate carcinomas from Canadian patients have been found to be positive for HPV DNA (8). The experimental observations demonstrating the binding of HPV E6-E7 oncoprotein with cellular tumor suppressor proteins (Rb and p53) and in vitro malignant transformation of E6-E7 immortalized human cells by additional transfection with v-ras oncogene clearly indicate the importance of other endogenous events besides HPV infection in these malignancies (7).

Previously we have studied HPV infections in normal and neoplastic lesions of the cervix (9) and urinary bladder (10). In this paper we report a relatively frequent occurrence of ras mutations and the presence of HPV DNAs in the prostatic carcinomas from 68 Japanese patients and discuss their possible roles in the pathogenesis of this cancer.

MATERIALS AND METHODS

Specimens. A variety of prostate carcinoma tissues from 68 patients with a mean age of 73 years (ranging from 53 to 88 years) were obtained from the Pathology Laboratory of Fukui Medical School Hospital and the Department of Pathology, School of Medicine, Mie University, Japan. The patients were divided into the following 4 groups on the basis of the sites from where the carcinoma tissues were taken for analysis: group I, 52 patients in whom the materials were obtained from the primary site of the tumor (Ia = latent carcinoma, n = 17; Ib = clinically diagnosed carcinomas, n = 35); group II, 10 patients in whom the materials were obtained from metastatic sites; group III, 5 patients in whom the materials were taken from primary as well as metastatic sites; and group IV, one patient with recurrent prostatic carcinoma in whom three biopsy specimens were taken at different intervals from the primary site. Staging of prostate carcinoma was performed according to the criteria proposed by Whitmore (11) and Jewett (12). Latent carcinoma was included in stage A in the present study. Ten specimens each of normal prostate obtained at autopsy and benign prostatic hyperplasia were also examined for comparison. The mean ages of the patients with normal and hyperplastic prostatic tissues were 63 (ranging from 46 to 78) and 69 (ranging from 58 to 80) years, respectively. Tissues had been fixed in 10% buffered formalin and embedded in paraffin. The paraffin blocks used in this study were stored at room temperature for 1—6 years. Hematoxylin and eosin-stained slides were prepared from these blocks. Histological grading of prostate adenocarcinoma was scored by the Gleason system (13).

Cell Lines and Recombinant HPV DNA. Four cell lines, EJ-1, LU-65, MOLT-4, and HL-60, having specific mutations at codon 12 of the H-ras, K-ras, and N-ras genes and at codon 61 of the N-ras gene, respectively, were used as positive controls. These cell lines, obtained from the Japanese Cancer Research Resources Bank, were maintained in vitro according to the instructions of the supplier. Recombinant viral DNAs of HPV 16, 18, and 33 were used as the target DNA for PCR.

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2 The abbreviations used are: HPV, human papillomavirus; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSPE (1× SSPE = 180 mm sodium chloride, 10 mm sodium dihydrogen phosphate, 1 mm EDTA, pH 7.4); Tm, melting temperature (°C).
amplification with their specific primers in control experiments as described previously (10).

DNA Extraction. DNA extraction was carried out according to the previously described method for formalin-fixed, paraffin-embedded tissues (10, 14). Briefly, the sections were deparaffinized twice with 1 ml of xylene and washed twice with ethanol. The residual ethanol was removed by rinsing the sections with acetone, and 100–200 μl of digestion buffer [50 mM Tris-HCl (pH 8.5), 1 mM EDTA, 0.5% Tween 20, and 200 μg/ml of proteinase K] were added. The mixture was incubated at 37°C overnight, and proteinase K was inactivated at 95°C for 10 min. The genomic DNA from the cell lines used as positive controls was prepared according to the established procedure (15).

Polymerase Chain Reaction. Five μl of the DNA solutions were used for PCR in a 50-μl reaction mixture containing 10 mM Tris-HCl (pH 8.9), 80 mM KCl, 1.5 mM MgCl2, 500 μg/ml bovine serum albumin, 0.1% Triton X-100, 0.1% sodium cholate, 80 μM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 1 μM of each primer, and 1.5 units of the Tth DNA polymerase prepared from a thermophilic bacteria *Thermus thermophilus* HB8 (Toyobo, Ltd., Osaka, Japan). The use of Tth DNA polymerase in PCR has already been reported (16). Templates were denatured at 95°C for 8 min before addition of the Tth polymerase at 72°C. This was followed by 35 cycles of PCR with incubations of 1 min at 50°C (annealing), 2 min at 72°C (polymerization), and 30 s at 95°C (denaturation). Negative controls were included in every set of reactions. To eliminate the possibility of contamination, the PCR reactions were segregated from post-PCR analysis, and positive displacement pipettors and a UV-irradiated biohazard hood were used. Twelve μl of PCR products were routinely checked for amplified DNA on 5% polyacrylamide gel.

**Synthetic Oligonucleotides.** All HPV and ras primers and HPV probes were synthesized on an ABI 381A DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) according to published sequences (6, 17, 18). The ras oncogene probe set was purchased from Takara Biomedicals (Kyoto, Japan). A complete list of oligonucleotide sequences for primers and probes is available on request. The oligonucleotides included in every set of reactions. To eliminate the possibility of contamination, the PCR reactions were segregated from post-PCR analysis, and positive displacement pipettors and a UV-irradiated biohazard hood were used. Twelve μl of PCR products were routinely checked for amplified DNA on 5% polyacrylamide gel.

**Oligonucleotide Probe Hybridization.** For the detection of ras mutation, nylon filters (Gene Screen Plus; Du Pont, NEN Research Products, Boston, MA) were prepared, and prehybridization and hybridization were performed as described by Verlaan-de Vries et al. (18) and Enomoto et al. (19), with some modifications. Briefly, the PCR products were denatured at 95°C for 7 min and were placed immediately on ice. Two μl of the denatured products were spotted onto the filters, and DNA was fixed by UV irradiation. The filters were prehybridized overnight at 56°C in a mixture of 3.0 mM tetramethyl ammonium chloride, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.5% SDS, 5× Denhardt's solution [0.5% Ficoll, 0.5% polyvinylpyrrolidone, 0.5% bovine serum albumin (Fraction V)], and 100 μg/ml of salmon sperm DNA. Hybridization was performed for 3 h at 56°C in the same mixture containing 32P-labeled probe (5× 106 cpd/ml, 1× 109 cpm/ng) together with an equimolar amount of the corresponding unlabeled wild-type probe, which decreases nonspecific binding.

The filters were sequentially washed as follows: two washes in 2× SSPE-0.1% SDS at room temperature for 20 min each and a wash in 5× SSPE-0.1% SDS for 30 min at 56°C. This was followed by a stringent wash for 20 min in 5× SSPE-0.1% SDS at Tm or Tm + 1°C of the corresponding probe. Tm was calculated by the empirical formula:

\[ Tm^{oC} = 4(G + C) + 2(A + T) \]

Finally, filters were rinsed twice in the hybridization buffer lacking Denhardt's solution and salmon sperm DNA and then incubated for 1 h in the same solution at 60°C.

For the detection of HPV, the preparation of filters and the protocol for prehybridization, hybridization, and washes were same as described (20). However, the prehybridization and hybridization were extended to 3 h, and a final stringent wash was performed at 60°C for 15 min. After the washes, the filters were dried and exposed to XAR film (Eastman Kodak Company, Rochester, NY) at −70°C for 1–24 h.

**Statistical Analysis.** Statistical analysis was performed using all parameters separately and in a multivariate analysis. Two-sided *P* values of less than 0.05 were considered statistically significant.

**RESULTS**

Seventy-five DNA samples from 68 patients with a variety of prostate carcinomas (as described in “Materials and Methods”) were screened for point mutations in codons 12 and 61 of the ras oncogenes (H-ras, K-ras, and N-ras) and for the presence of high-risk HPVs (types 16, 18, and 33). The stage of the prostate carcinomas varied from stage A (including 17 cases of latent carcinomas) to D, and the Gleason scores varied from 2 to 9 (Table 1). Of these 68 cases, 16 (24%) contained ras mutations. The majority of ras mutations were restricted to codon 61 of the H-ras (11 of 16 cases, 69%) (Fig. 1). Four mutations were detected at codon 12 of the N-ras and 2 mutations at codon 61 of the K-ras. None of the samples showed mutations in codon 12 of the H- and K-ras and in codon 61 of the N-ras. One case...
The correlation between the presence of ras mutation and HPV DNA, and the stage and Gleason score of the tumor, is summarized in Table 2. Both ras mutation and HPV infection were more frequent in the tumors in stages C and D than in stages A and B. Similarly, the same trend was observed with the Gleason score of the tumor. There was no difference in the rate of ras mutation and HPV infection between the tumors metastatic to the bone (29% and 54%) or those metastatic to other sites including lung, liver, brain, etc. (22% and 56%). However, the ras mutations were restricted to codon 61 of the H-ras in 7 (88%) of 8 tumors with bone metastasis. Moreover, HPV 18 infection was observed in 12 (80%) of 15 HPV-positive cases (80%) with bone metastasis (Table 3). The mean ages of the patients having ras mutation and HPV DNA were 74 and 73 years, respectively.

None of the normal or benign hyperplastic prostatic tissues (10 of each) were found to contain either any ras mutation or the high-risk HPV DNA.

DISCUSSION

Carcinogenesis is a complex multistep process involving a number of genetic and epigenetic events in protooncogenes, tumor suppressor genes, and antimetastasis genes (21). Mutations in ras oncogenes are not uncommon in rodent cancers induced by exposure to a variety of carcinogens (22). Similarly, ras mutations have been detected in a wide variety of human neoplasms, but the frequency of mutational activation varies greatly, depending upon tumor type (23). Previous studies have reported that the incidence of ras mutation is rare in urogenital malignancies including prostate carcinoma, with one exception of urinary bladder carcinoma (5, 6, 23, 24). However, recent experimental data have raised the possibility of the involvement of ras and myc oncogenes in inducing malignancy when introduced into the cells forming a reconstituted mouse prostate gland (25). Likewise, there is substantial experimental evidence suggesting an etiological role of high-risk HPV, especially in anogenital carcinogenesis (7).

Using a sensitive technique of PCR amplification of target DNA sequences followed by hybridization analysis with specific...
Americans. A review of prostate carcinomas in autopsy cases, et al. (26) reported the same trend with prostatic carcinoma in white and 32—37% of black patients, respectively (26). The contrast to 20% of the 130 cases in white patients from Honolulu, Hawaii (collected from one hospital during 1976—1986).

In a large-scale study from the United States, the frequency of well-differentiated carcinoma compared to stage D prostatic carcinoma was reported to be 20—24% of prostate carcinomas in Japanese patients are in late stages at the time of diagnosis. An international comparative study of prostatic carcinoma by Yatani et al. (3) showed that 42% of the 693 cases of prostate carcinomas in Japanese patients (collected from five different cities during 1966—1981) were diagnosed in stage D in contrast to 20% of the 130 cases in white patients from Honolulu, Hawaii (collected from one hospital during 1976—1986).

In a large-scale study from the United States, the frequency of stage D prostatic carcinoma was reported to be 20—24% of white and 32—37% of black patients, respectively (26). The biological behavior of clinically manifest prostate carcinoma is far more aggressive in Japanese patients than in patients from the United States (3, 26). Well-differentiated carcinoma comprised 16% of a total of 693 prostate carcinomas from Japan as compared with 29% of the 207 cases from Hawaii (3). Murphy et al. (26) reported the same trend with prostatic carcinoma in Americans. A review of prostate carcinomas in autopsy cases, comprising 136 Japanese cases reported in “The 1980s Annual of the Pathological Autopsy Cases in Japan” and 576 cases from the United States reported by Veterans Administration Cooperative Urological Research Group (Gleason et al. J. Urol. 1974), revealed that distant metastatic lesions were 2 to 5 times more common in Japanese cases as compared with Americans (4). The mortality:incidence ratios are also substantially higher in Japan than in the United States (27).

The frequency of ras mutation doubled in stages C and D (28%) as compared with stages A and B (14%), as shown in Table 2. Our results support the hypothesis that ras mutations are not an obligatory early step and may arise during any stage of carcinogenesis but occur more commonly in the progression stage of established malignancy. The ras mutation may play a role in the induction of the metastatic potential of the tumor. There is circumstantial evidence that the activation of certain oncogenes in certain tumors may be associated with progression to a more malignant phenotype (28). Moreover, it has been demonstrated that the invasive phenotype of human transitional cell carcinoma is induced by the overexpression of either a mutated or normal H-ras, when it is inoculated intravesically into nude mice (29). Treiger and Isaacs (30) have demonstrated that the expression of an activated H-ras oncogene can confer high metastatic ability on a tumorigenic but nonmetastatic Dunning rat prostate adenocarcinoma cell line, AT2.1. The frequency of ras mutation increased in patients with prostate carcinoma having higher Gleason scores (Table 2). A study by Viola et al. (31) also showed a good correlation between the expression of Ras p21 protein as assessed by immunohistochemistry and the histological grade of prostate carcinoma. The clinical correlation of the Gleason score with other prognostic factors including metastasis is well established (1). Our present findings, therefore, suggest that ras mutations are somehow associated with the extensive growth and metastatic potential of this tumor.

It should be noted that the majority of the mutations (11 of 16, 69%) were in H-ras codon 61,2, corresponding to a glutamine to leucine substitution (A→T transversion). Although the clinical significance of this particular type of ras mutation is unknown at present, the same mutation has been found to be 16% in 627 cases of prostate carcinomas in Japanese patients. A possible explanation for this high frequency may be the fact that most prostatic carcinomas in Japanese patients are in late stages at the time of diagnosis. An international comparative study of prostatic carcinoma by Yatani et al. (3) showed that 42% of the 693 cases of prostate carcinomas in Japanese patients (collected from five different cities during 1966—1981) were diagnosed in stage D in contrast to 20% of the 130 cases in white patients from Honolulu, Hawaii (collected from one hospital during 1976—1986). In a large-scale study from the United States, the frequency of stage D prostatic carcinoma was reported to be 20—24% of white and 32—37% of black patients, respectively (26). The biological behavior of clinically manifest prostate carcinoma is far more aggressive in Japanese patients than in patients from the United States (3, 26). Well-differentiated carcinoma comprised 16% of a total of 693 prostate carcinomas from Japan as compared with 29% of the 207 cases from Hawaii (3). Murphy et al. (26) reported the same trend with prostatic carcinoma in Americans. A review of prostate carcinomas in autopsy cases, comprising 136 Japanese cases reported in “The 1980s Annual of the Pathological Autopsy Cases in Japan” and 576 cases from the United States reported by Veterans Administration Cooperative Urological Research Group (Gleason et al. J. Urol. 1974), revealed that distant metastatic lesions were 2 to 5 times more common in Japanese cases as compared with Americans (4). The mortality:incidence ratios are also substantially higher in Japan than in the United States (27).
reported in rodent stomach and lung carcinomas induced by aristolochic acid (32).

The prevalence of the HPV infection in Japanese prostate carcinoma in this study is almost the same as that reported in Canadian patients (8). The importance of HPV in prostatic neoplasia is further strengthened by our current observation that HPV DNA was not detected in any of the normal or hyperplastic prostatic tissues. The presence of HPV in 8 (50%) of 16 cases of the specimens containing ras mutation also suggests the possible role of HPV in prostate carcinogenesis, because there is already experimental evidence that HPV 16 can transform primary human fibroblast cells in the presence of activated EJ-ras (33). Such a high incidence of HPV infection in prostate carcinoma may not be unexpected, since there is some analogy between prostate and cervical carcinoma. Previous reports of prostate carcinoma have cited the importance of multiple sexual partners, frequent intercourse, contact with prostitutes, and history of genital infections in wives of the patients with prostate carcinoma (34). It has been reported that tissue contamination by virally infected urethral cells may confound HPV analysis of prostate cancer (35). In the present study, however, only 6 specimens were found to contain the urethral mucosa, and all of these specimens except one were negative for HPV DNA.

Although we did not use Southern hybridization, the detection of HPV DNA in 7 metastatic prostatic carcinomas may suggest that the HPV DNA may be in the integrated form at least in these cases. In one previous study both episomal and integrated HPV sequences were observed in benign and malignant tumors of the prostate (36). Further studies regarding the integration of this virus in neoplastic tissues will clarify the role of HPV infection in prostate carcinogenesis.

We showed that HPV positivity was significantly higher in stages C and D (55%) as compared with stages A and B (10%) (P < 0.0005). Similarly, the HPV infection rate also increased significantly with increasing Gleason score (P < 0.005). These results clearly suggest the importance of HPV infections in the later stages of prostate carcinogenesis.

There was no difference in the distribution of ras mutation and HPV infection between prostatic carcinomas metastatic to the bone and those metastatic to other organs. However, in all cases but one, the ras mutations in tumors with bone metastasis were restricted to one particular type, i.e., H-ras codon 61.2. This fact seems to be unique and may have some predictive value for the metastatic potential of prostatic carcinoma to the bone. Similar findings have been reported by Fan (37), who suggested that immunohistochemical evaluation of the Ras protein, p21, in the primary tumor cell population may be clinically useful as a predictor for the metastatic potential of human prostatic tumors. Likewise, we found that HPV 18 infection was common (12 of 15 HPV-positive cases, 80%) in prostatic carcinomas with bone metastasis. Whether the infection with a particular type of HPV is associated with preferential metastasis to certain organs is presently not known. However, infection with HPV 18 in cervical carcinoma has been reported to follow a more aggressive course, with a propensity for rapid metastasis and high mortality (38).

In summary, the overall frequency of ras mutations and HPV infection, at least in Japanese patients with (mostly advanced) prostate carcinoma, is relatively high. This suggests that racial, environmental, and other factors are involved in prostate carcinogenesis. Future studies from different geographical regions are needed to further clarify these observations. Moreover, H-ras codon 61.2 mutation and HPV 18 infection may be useful as predictors for bone metastasis.

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