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Histochemical Examination of Expression of ras p21 Protein and R 1881-binding Protein in Human Prostatic Cancers

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Expression of ras p21 was examined with monoclonal antibody RASK-3 in normal, benign hyperplasic, and cancerous prostates. In patients with stage D2 disease who received endocrine therapy, the relation between ras p21 expression, response to therapy, and prognosis was studied. In these patients, R 1881-binding protein (androgen receptor and progestin-binding protein) was also examined. Non-cancerous cells and most cancer cells from stage A patients did not express ras p21, while expression increased with both higher staging and grading. Staging pelvic lymphadenectomy was done in some stage A2-C cases, and presence of nodal metastasis was correlated with ras p21 expressions in the primary tumours. In stage D2, there was no correlation between ras p21 expression and R 1881-binding protein. Response to therapy and survival did not correlate with expression of ras p21, but was influenced by presence of R 1881-binding protein.
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INTRODUCTION

RAS p21, the protein coded by *ras* gene, has been observed in various human cancers including prostate [1]. Although increased expression of *ras* gene is related to rapid and invasive growth [2], the significance of this finding is not fully known. Most prostatic cancers initially respond to endocrine therapy; susceptibility is related to high levels of dihydrotestosterone and androgen receptor [3]. Previously we found that the amount of R 1881-binding protein (androgen receptor and progestin-binding protein) in the tissues correlated with response to endocrine therapy and also to prognosis of stage D2 prostatic cancers [4]. Here we have examined ras p21 expression, and the R 1881-binding protein in prostatic cancers related to response to endocrine therapy and survival.

PATIENTS AND METHODS

Patients

Patients with prostatic cancer admitted to Chiba University Hospital were studied. Prostatic cancer tissue was obtained after perineal biopsy (stage B–D, before treatment) or transurethral resection (TUR; operated on for benign prostatic hyperplasia, stage A). Clinical staging by the US system [5], pN classification by UICC, and histological grading by Gleason [6] were used, and primary patterns of I and II, III, and IV and V were classified as well, moderately, and poorly differentiated carcinomas, respectively.

Endocrine therapy was given as the first treatment to 62 patients with stage D2 tumours. 47 patients were treated by orchiectomy and the immediate administration of a daily dose of 250 mg diethylstilbestrol diphosphate for 4 weeks, followed by a daily dose of 1–1.5 mg ethynyl-oestradiol until relapse became evident. If cardiovascular disorder occurred, a daily dose of 100 mg chlormadinone was used instead of oestrogen after orchiectomy (15 cases). Response to endocrine therapy was evaluated at 6 months after the start of treatment [7]. Prognosis was calculated by cause-specific actuarial survival [8].

Staining of ras p21

A monoclonal mouse antibody against ras p21, designated as RASK-3, was donated by Dr H. Shiku. This antibody reacted with Ki-, N-, and Ha-ras p21 [9]. Tissues were fixed in buffered formalin and 3 μm paraffin sections were prepared. After removal of paraffin, the sections were passed through methanol containing 0.3% hydrogen peroxide for 30 min, then incubated with 5% normal horse serum for 30 min. RASK-3 was applied to the sections at 37°C for 1 h, then left at room temperature overnight followed by sequential treatments with biotinylated horse anti-mouse IgG antiserum, ABC kit and DAB staining (Vector Labs, Burlingame, California). As controls, sections of normal or benign hyperplasic human prostates were stained in parallel. A slight and even staining in the cytoplasm of epithelium of the gland from normal and hyperplasic prostates was consistently noticed, and this degree of staining was designated negative. Cases showing more than 10% of cancer cells with strong staining were referred to as positive.

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Table 1. Expression of ras p21 in normal, benign hyperplasic, and cancerous prostates

	ras p21	
	Positive	Negative
Normal prostate (n = 12)	0	12
Benign prostatic hyperplasia (n = 64)	0	64
Incidental carcinoma (n = 62)		
Well differentiated	0	42
Moderately differentiated	3 (17%)	15
Poorly differentiated	0	2
Stages B–D (n = 121)*		
Well differentiated	2 (22%)	7
Moderately differentiated	34 (59%)	24
Poorly differentiated	42 (78%)	12

*Comparisons between positive and negative: well *vs* moderate (P = 0.046 by Fisher's exact test), well *vs* poor (P = 0.002), moderate *vs* poor ($P < 0.05, \chi^2$), well *vs* moderate plus poor (P = 0.021), well plus moderate *vs* poor ($P < 0.01, \chi^2$).

R 1881-binding protein

Frozen tissue sections were used for staining of R 1881-binding protein [10]. For positive controls, sections of normal or hyperplasic prostates were used. More than 10% staining of cells was designated positive.

RESULTS

Ras p21 expression

Intense staining of ras p21 was not observed either in normal or in hyperplasic prostates (Table 1). In positive cancer cells, cytoplasm was diffusely stained, but ras p21 expression was not detected in the nucleus (Fig. 1). Stroma was not stained. In incidental cancers, stage A1 did not show positive staining, but 3 out of 32 stage A2 specimens were positive and positive cancers were moderately differentiated carcinomas. Clinical cancers including 2 stage B, 17 C, 26 D1, and 76 D2 were examined. 2 stage B, a well and a moderately differentiated carcinoma, were negative. Ras p21 positive tumours in stage C and D1 were, respectively, 0/2 and 2/6 in well differentiated, 3/8 and 6/11 in moderately differentiated, and 4/7 and 8/9 in poorly differentiated carcinomas. In stage D2, 25/38 and 30/38 were positive in moderately and poorly differentiated carcinomas, respectively.

39 patients (5, 1, 7, and 26 in stage A2, B, C and D1, respectively) underwent staging pelvic lymphadenectomy. Expression of ras p21 in cancer cells of the prostate correlated with the presence of nodal metastasis (Table 2). pN1, pN2 pN3, and pM_1LYM in ras p21 positive and negative cases were 2, 8, 2, 4 and 4, 5, 0, 1, respectively. Positive expression of ras p21 seemed to promote an advanced extent of metastasis, but no statistical difference in nodal status was noticed between ras positive and negative N+ cases. These results indicated that expression of ras p21 increased with progression of the stage and grade.

*Table 2. Relation between expression of ras p21 in cancer cells of prostate and occurrence of regional lymph node metastasis**

	ras p21	
	Positive	Negative
Node metastasis		
Present	16	10
Absent	3	10

*P = 0.026, Fisher's test.

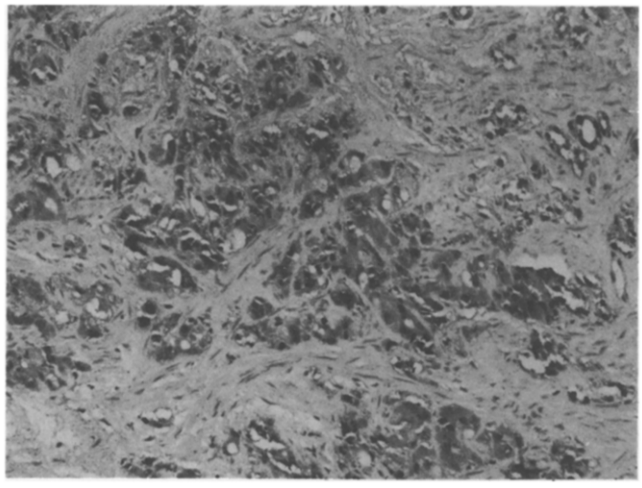

Fig. 1. Staining with RASK-3 of moderately differentiated adenocarcinoma, showing diffuse expression of the oncogene.

R 1881-binding protein

Patients in stage D2 were examined for expression of ras p21 and R 1881-binding protein before the endocrine therapy. Ras p21 did not correlate with positive staining for R 1881-binding protein (Table 3).

ras p21, R 1881-binding protein, and response

4 out of 62 stage D2 patients who received endocrine therapy were unsuitable for evaluation of the effect at 6 months after the start of therapy. In 58 cases the relation between effect and expression of ras p21 or R 1881-binding protein was examined (Table 4). There was no significant relation between ras p21 and response to the endocrine therapy. However, the presence of R 1881-binding protein was correlated with response to therapy, which was similar to the results reported previously [4].

Cause-specific actuarial survival of these 62 cases did not correlate with expression of ras p21, while there was a relation between the survival and the presence of R 1881-binding protein (Fig. 2). These results indicated that the expression of R 1881-binding protein was a useful factor to predict survival after endocrine therapy in advanced prostatic cancer.

*Table 3. Relation between expression of ras p21 and R 1881-binding protein in cancer cells of prostates from patients with stage D2 prostatic cancer**

	ras p21	
	Positive	Negative
R 1881-binding protein		
Present	20	11
Absent	22	9

*Not significant by Fisher's test.

Table 4. Expression of ras p21 and R 1881-binding protein in relation to response to endocrine therapy 6 months after start of treatment

	ras p21*		R 1881-binding protein†	
	Positive	Negative	Present	Absent
PR[a]	32	8	26	14
OS	2	5	3	4
PD	5	6	1	10

a: PR = partial response, OS = objectively stable, PD = progressive disease.
*Frequency of presence of ras p21 (PR+OS)PD: P not significant by Fisher's test.
†Frequency of presence of R 1881-binding protein: $P = 0.002$, Fisher's test.

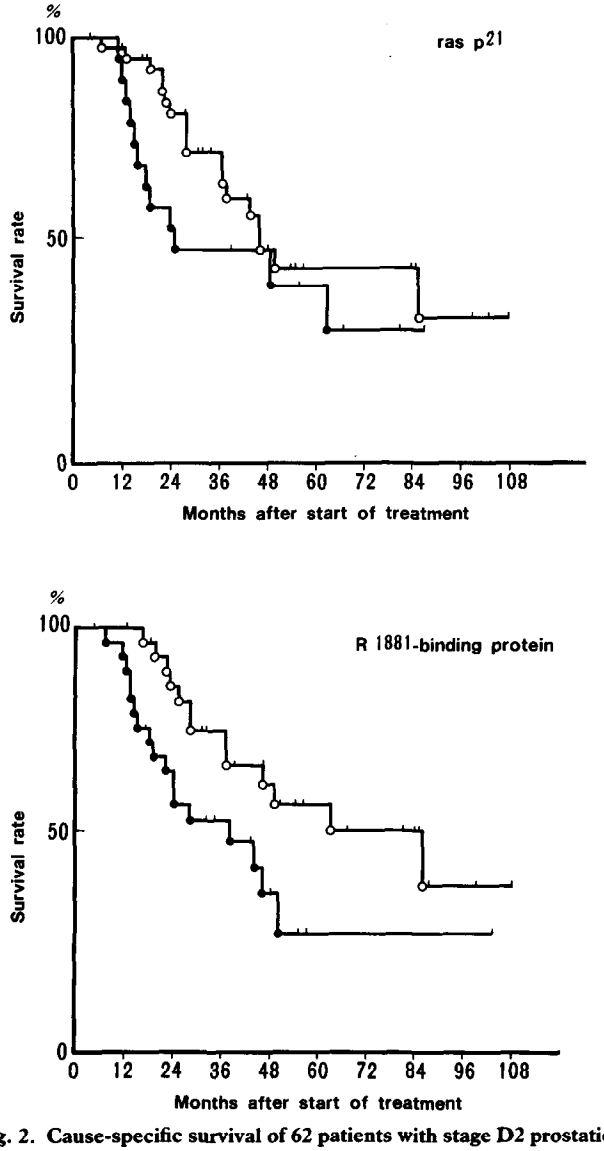

Fig. 2. Cause-specific survival of 62 patients with stage D2 prostatic cancer who received endocrine therapy. Upper = as function of expression of ras p21, open circles = positive, closed circles = negative. Not significant by generalized Wilcoxon test. Lower = R 1881-binding protein, open circles = present, closed circles = absent; $P < 0.05$.

DISCUSSION

Increased expression of ras p21 has been observed in various human cancers, such as stomach [11, 12], colon [13, 14], lung [15], ovary [16], thyroid [17], breast [18], bladder [19], and neuroblastoma [20]. It has generally been accepted that cancers of low differentiation with a high ability to invade with nodal metastasis show high expression of ras p21, and that ras 21 may be one of the factors contributing to the malignant phenotypes.

In human prostatic cancer, increased expression of some oncogenes is frequently observed. Increased c-myc expression was reported in various cancer tissues and also in cultured cell lines [21–23]. Transfection of DNA isolated from prostatic cancers into 3T3 cells induced expression of Ki-*ras* oncogene, suggesting that some prostatic cancers contained this gene amplification [24]. ras p21 expression examined immunohistochemically was dependent on the grade of clinical prostatic cancer: 33, 67, and 100% showed positive expression in grades 1, 2, and 3, respectively [25]. Although the rate was different, we found ras p21 expression to be dependent on grade, and the rate of positive expression was increased with progression of clinical stage within the same grade. Cancer cells exhibiting positive ras p21 expression tended to have a higher frequency of nodal metastasis (Table 2). In this context, ras p21 was suggested as a predictor for bone metastasis [26]. Expression of ras p21 was rarely observed in stage A (Table 1); it is accepted that cancer cells in stage A are in a dormant state. Expression of ras p21 was not correlated with the survival of stage D2 (Fig. 2A), which may be explained by the fact that ras p21 positive cancers initially responded to endocrine therapy similarly to ras p21 negative cancers. Expression of ras transcript was reduced in the androgen-sensitive PC 82 cells after withdrawal of androgen [22]. Thus the effect of endocrine therapy may overcome the malignant property, if any, of prostatic cancer cells associated with expression of *ras* oncogene.

Since ras p21 examined with RAP-5 or Y13-259 showed positive staining on normal mucosa and also premalignant and malignant tumours of the colo-rectum, thyroid, and mammary gland [27–30], it was claimed that the expression of ras p21 was not a useful measure for detecting malignant phenotypes. In the human prostate, the ras product detected with an antibody against a synthetic peptide corresponding to amino acid positions 160–179 of Ha-ras p21 was widely distributed in benign as well as in malignant epithelium [31]. Therefore, an antibody raised against ras p21 for detection of the oncogene product is important. Different results were obtained with RAP-5 or Y13-259 to distinguish hamster fibroblasts with Ha-ras insertion from those without oncogene expression [32]. We used a monoclonal antibody against ras MW 21 000 protein (RASK-3), which stained highly positively in stomach and thyroid cancers while the intensity of staining in non-cancerous tissues was weak. With this antibody, normal and benign hyperplasic prostatic epithelium were weakly stained. Thus it is important to compare benign and malignant tissues at the same time. Although expression of ras p21 seems to be the first step during the course of malignant transformation [33–35], ras p21 is one of the oncogenes whose expression is increased in prostatic cancers. Therefore examination of its expression might be helpful in understanding the state of malignancy of the prostatic tumour cells.

In breast cancers, a relation between estrogen receptor and ras p21 expression has been reported [36], but these observations have been controversial. We found no correlation between ras p21 expression and presence of R 1881-binding protein in

prostatic cancers. Thus it might be suggested that expression of *ras* oncogene in sex hormone sensitive cancers is independent of hormonal regulations. In this context, the presence of the nuclear androgen receptor in prostatic cancers was not correlated with mRNA levels of c-*myc*, H-*ras* and K-*ras*, but was correlated with that of c-*fos* [37]. On the contrary, ras p21 protein content in hormone-dependent mammary cancers of human and rat was about 7-fold higher than that of hormone-independent cancers [38]. An inverse relation between *erb* B-2 expression and oestrogen receptor content has been reported in mammary cancers [39]. Whether or not increased expression of oncogenes in hormone-responsive cancers is linked to hormonal regulation is an important question.

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