Jpn. J. Cancer Res. **91**, 941–947, September 2000

p53 Mutations in Prostatic Intraepithelial Neoplasia and Concurrent Carcinoma: Analysis of Laser Capture Microdissected Specimens from Non-transition and Transition Zones

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Prostatic intraepithelial neoplasia (PIN) is characterized by intraluminal proliferation of epithelial cells and is divided into high-grade (HGPIN) and low-grade (LGPIN) lesions. HGPIN is regarded as the most likely precursor of prostatic cancer (PCA). Microdissected DNA selectively extracted from paraffin-embedded sections of 27 cases with PCA were analyzed for *p53* mutation in exons 5–8 by single-strand conformation polymorphism of polymerase chain reaction-amplified DNA fragments (PCR-SSCP) followed by direct sequencing. These patients received total prostatectomy (27 cases). After a review of histologic sections, DNA was extracted from 193 locations; 111 lesions from 27 cases with HGPIN (75 lesions from non-transition zone and 36 from transition zone), 55 lesions from 27 cases with PCA (30 lesions from non-transition zone and 25 from transition zone), and 27 from 27 benign glands. Analysis revealed 27 mutations of the *p53* gene in 24 lesions from 12 cases. Benign glands adjoining PIN and/or PCA had no mutations. All mutations were point mutations: 17 missense, 7 silent, and 2 nonsense. Mutations were detected in 6 cases (22.2%) or 13 of 111 lesions (11.7%) with HGPIN and 8 cases (29.6%) or 11 of 55 lesions (20.0%) with PCA. In a given case, HGPIN and PCA lesions had different *p53* mutations from each other, suggesting multiclonal development of prostatic precancerous lesions. The frequency of *p53* mutation of PCA was significantly higher in the non-transition zone (33.3%) than in the transition zone (4%), and higher in the stage T3 cases (30.3%) than in the stage T2 cases (4.5%, 1 of 22 lesions) (both $P<0.05$). Frequency of *p53* mutation of PIN in the non-transition zone (14.7%) was higher than that in the transition zone (5.6%), although the difference was not significant. The frequency rate of *p53* mutation in HGPIN close to PCA (≤2 mm) was significantly higher (24%) than that in HGPIN lesions >2 mm from PCA (3%). All these findings indicate that the *p53* gene mutations are involved in prostatic carcinogenesis and explain why the non-transition zone is the predominant site of PCA.

Key words: Prostatic intraepithelial neoplasia — Prostatic cancer — *p53* — PCR-SSCP

Prostatic intraepithelial neoplasia (PIN) is characterized by an intraluminar proliferation of epithelial cells in ducts and acini. PIN frequently coexists with prostatic carcinoma (PCA),[1–3] is usually multicentric, and is commonly found in the non-transition zone, which is the predominant site of PCA.[4, 5] According to the histologic and cytologic findings, PIN can be divided into high-grade PIN (HGPIN) and low-grade PIN (LGPIN).[6] Previous histopathological study on whole-mount prostatectomy specimens showed that HGPIN lesions were detected in more than 75% cases with PCA.[7, 8] Proximity of PCA and PIN (≤2 mm) was proposed to indicate a precancerous nature of PIN,[7] and our recent study[8] revealed that close association of PIN with PCA was more frequently found in HGPIN (54% of lesions) than in LGPIN (38%). All these findings indicated that HGPIN, but not LGPIN, is the most likely precursor lesion for PCA.

Recent studies revealed that HGPIN and PCA share common cytogenetical features. Allelic loss of chromosome 8p was frequent both in PIN and invasive PCA.[9, 10] Mutations of *Ha-ras* gene were closely associated with progression of PIN into invasive PCA in transgenic mice, although this was not confirmed in humans.[11]

The *p53* gene is a tumor suppressor gene located on the short arm of chromosome 17. It consists of 11 exons and 10 introns and encodes 393 amino acids.[12, 13] In a wide variety of human tumors, *p53* gene mutations have been detected mainly in exons 5 through 8, which include the highly conserved domains II–V.[14] Little is known about *p53* mutations in PIN due to technical problems, i.e., it is difficult to excise exclusively the PIN lesions, which are fairly small and difficult to define macroscopically. HGPIN frequently coexist with cancer or benign glands in

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a haphazard fashion in the non-transition zone. Recently we successfully microdissected PIN and PCA lesions, and examined *p53* mutations in PIN and PCA.[15]

Laser capture microdissection under direct microscopic visualization enables rapid one-step procurement of selected human cell populations from a histologic section. This method has made microdissection of selected areas much easier, so that extensive study of specific target lesions is possible. Using this method on the whole-mount samples, we have now selectively microdissected numerous lesions of PIN (both high and low grade) and PCA from the non-transition and transition zones. DNA extracted from each lesion was analyzed for *p53* mutations by single-strand conformation polymorphism (SSCP) of polymerase chain reaction (PCR)-amplified DNA fragments, followed by direct sequencing.

MATERIALS AND METHODS

Patients Twenty-seven patients, who received total prostatectomy for PCA, were selected for the current study: they had been admitted to hospital during the period 1996 to 1998. Fourteen patients received androgen deprivation therapy (castrated): an LH-RH agonist (leuprolide) for 2 to 6 months (mean 3.4 months) together with the anti-androgen agents (flutamide or chlormadinone), and the remaining 13 did not (non-castrated). Then the current cases were conventionally subdivided into preoperatively non-castrated group (13 cases) and medically castrated group (14 cases). None of the 27 patients received preoperative chemotherapy or radiation therapy. Based on the American staging system (modified by Whitmore-Jewett),[16] pathological stage was determined as follows: 11 cases (41%) in stage T2 and 16 (59%) in T3. Histologic specimens were fixed in 10% neutral buffered formalin and routinely processed for paraffin-embedding. Serial 5-μm-thick sections were cut, and every first section was stained with hematoxylin and eosin for histologic diagnosis. Mean number of sections examined was 9.3 per case. Histologic sections were reviewed independently by three pathologists (H.T., M.S. and K.A.) (Fig. 1). In problematical cases, preservation of the basal cell layer, a reliable criterion for benign prostatic lesions, was immunohistochemically examined by using basal cell-specific, high-molecular-weight cytokeratin antibody (34βE12). PIN is characterized by the intraluminar proliferation of crowded and stratified cells. HGPIN have features mimicking PCA, i.e., enlargement of the nuclei and the prominent nucleoli as well as a partially disrupted basal layer.

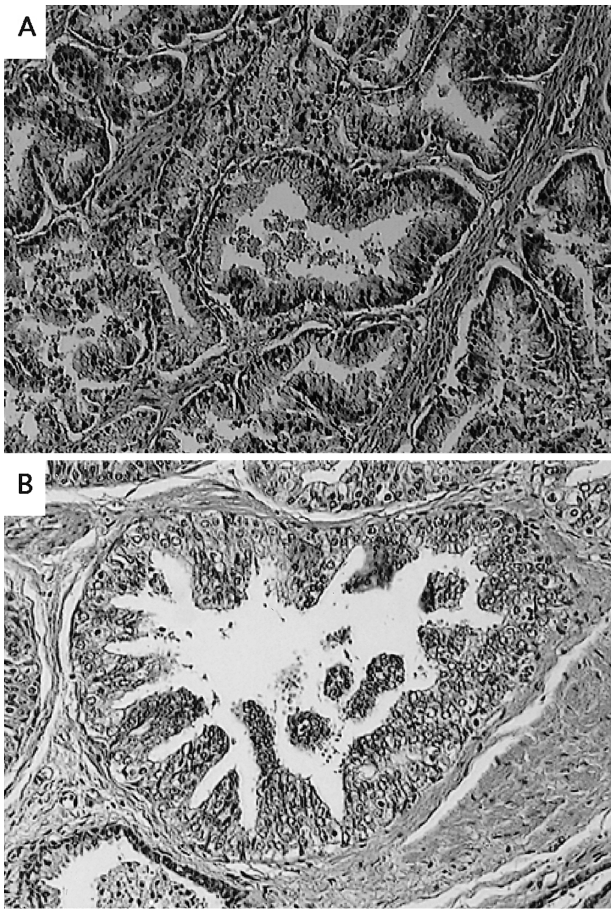

Fig. 1. Representative cases of prostatic carcinoma (Gleason pattern 5=2+3, grade II) (A) and high-grade prostatic intraepithelial neoplasia (B). (Hematoxylin and eosin staining, ×200)

Laser capture microdissection and DNA extraction Microdissection of each lesion was performed using a PixCell laser capture microscope (Arcturus Engineering, Santa Clara, CA) according to the previously described methods with some modifications[17, 18]; a representative case is shown in Fig. 2. Briefly, histologic sections were dehydrated, and then the histologic fields of interest were selected, overlaid with a thermoplastic film mounted on a transparent cap, and captured by the film through laser energy. The dissected pieces were allowed to adhere to the transparent cap and collected in 0.5 ml Eppendorf tubes. The procured cells were subsequently resuspended in 20–50 μl of extraction buffer containing 10 m*M* Tris (pH 8.0), 2 m*M* EDTA, 0.2% Tween 20, and 200 μg/ml proteinase K, and incubated overnight at 37°C. The mixture was heated at 100°C for 10 min to inactivate the proteinase K, and 3–5% of the solution was used as a template for each PCR. The total number of microdissected lesions from 27 cases was 193; 111 lesions with HGPIN (75 lesions from non-transition and 36 from transition zone), 55 with PCA (30 lesions from non-transition and 25 from transition zone), and 27 with benign glands.

PCR-SSCP analysis and direct sequencing The proce-

dure was carried out as previously described,[19] with a minor modification to increase efficiency; (1) the amount of microdissected DNA template for PCR amplification was 1 μl, (2) possible mutated bands at SSCP were extracted from gels and incubated with 20 μl of diffusion buffer containing 50 m*M* Tris-HCl, 1 m*M* EDTA, and 0.5% Tween 20 at 50°C for 1 h to make templates for subsequent amplification, (3) PCR products were extracted from 2% agarose TBE (Tris-borate, EDTA) gels. Mutations of *p53* gene from exon 5 to exon 8 were analyzed.

RESULTS

As shown in Table I, 27 mutations of the *p53* gene were detected in 24 lesions from 12 cases (Fig. 3). All mutations were point mutations; 17 were missense, 7 silent, and 2 nonsense. There were no mutational hot spots, although exon 5 was the commonest site. In cases 3, 19 and 20, there were double mutations with different types of nucleotide substitutions in the same exons. Mutations were detected in 6 cases (22.2%) or 13 of 111 lesions (11.7%) with HGPIN and 8 cases (29.6%) or 11 of 55 lesions (20.0%) with PCA. Benign proliferative glands adjoining PIN and/or PCA had no mutations of the *p53* gene. The PCA cases with mutations had stage T2 (2 cases) and T3 (6 cases) disease. In cases 19 and 20, two each of the PCA and PIN lesions had different mutations. In cases 6, 16, 17, 18 and 21, each of the HGPIN and PCA lesions had different mutations from each other. Mutations at CpG sites were found in one case (case 21). Regarding patterns of *p53* mutations, G-to-A transition was the commonest pattern (6/27; 22.2%), followed by C-to-T transition (5 mutations) and A-to-C transversion (5 mutations).

The frequency of *p53* mutation of PCA in the non-transition zone (33.3%) was significantly higher than that in the transition zone (4%) ($P<0.05$) (Table II). The frequency of *p53* mutation in PCA with stage T3 (30.3%), 10 of 33 lesions, was significantly higher than that with stage T2 (4.5%), 1 of 22 lesions ($P<0.05$). The frequency of *p53* mutation of PIN in the non-transition zone (14.7%) was higher than that in the transition zone (5.6%), although the difference was not significant. The frequency rate of *p53* mutation in HGPIN close to PCA (≤2 mm of distance) was significantly higher (24%) than that in HGPIN lesions >2 mm from PCA (3%) ($P<0.05$).

When *p53* mutations were evaluated in non-castrated cases (Table III), the mutation frequency of PIN was the same as that of PCA, i.e., 4 of 13 cases (30.8%). Five of 19 lesions with PCA and 8 of 36 lesions with PIN in the non-transition zone showed mutation, but none of PCA and PIN in the transition zone did. Nine of 31 HGPIN lesions close to PCA (≤2 mm) showed mutations, but none of HGPIN >2 mm from PCA did.

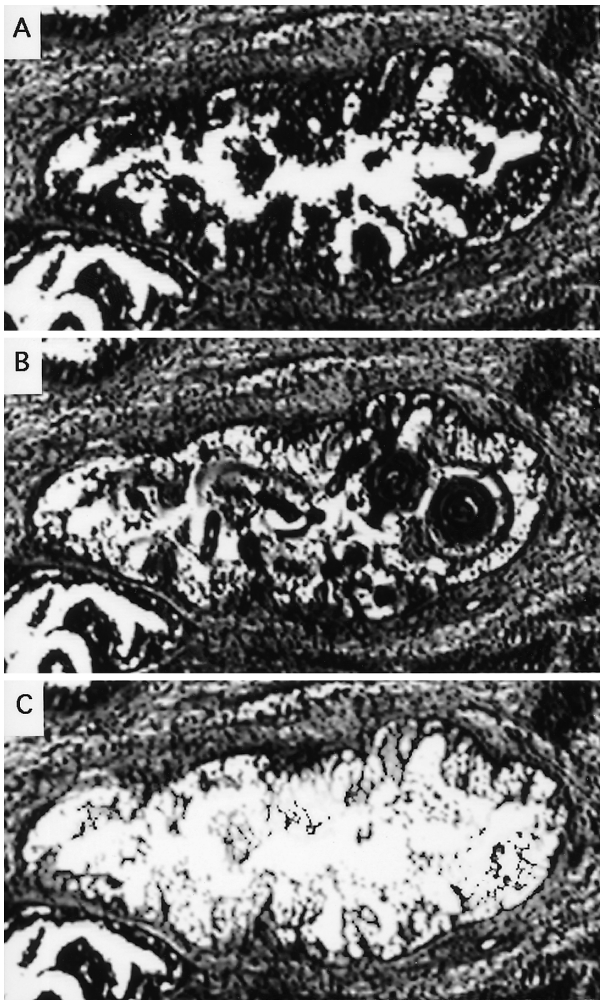

Fig. 2. Sequential laser capture microdissection of PIN in a representative case is shown before (A), during (B), and after (C) the microdissection procedure.

DISCUSSION

The current series consisted of 14 castrated and 13 non-castrated patients. The mutation frequency was similar in the castrated and non-castrated group, so they are discussed below as a total. The frequency rate of *p53* gene mutations in the current PCA lesions with mostly stage T2 or T3 disease (20%) was close to that in our previous study (17%)[15] and also that in reported cases with advanced PCA.[20–22] *P53* gene mutations were found in 12% of the current HGPIN lesions, which is also similar to that of our previous study (9%).[15]

Difference in mutation frequency by stage of PCA could not be evaluated in our previous study because of the relatively small number of PCA lesions examined.[15] In

Table I. *p53* Mutations in PIN and Concurrent PCA

Case	Zone of lesion	Distance from PCA (mm)	Histology/pathological stage	Exon/codon	Mutation	Nucleotide substitution	Pattern
Castrated group (*n*=14)							
3	N-T		PCA/Gleason 2, T3	7/235	Asp (AAC) → His (CAC)	A to C	Tv missense
				7/237	Met (ATG) → Arg (AGG)	T to G	Tv missense
18	N-T	1	HGPIN	8/285	Glu (GAG) → Stop (TAG)	G to T	Tv nonsense
	N-T	1	HGPIN	5/145	Leu (CTG) → Leu (CTA)	G to A	Ts silent
21	N-T	0	HGPIN	5/151	Pro (CCC) → Pro (CCG)	C to G	Tv silent
	T	13	HGPIN	5/165	Gln (CAG) → Gln (CAA)	G to A	Ts silent
	T	13	HGPIN	5/158[a)]	Arg (CGC) → Asp (CAC)	G to A	Ts missense
24	T		PCA/Gleason 2, T2	8/285	Glu (GAG) → Stop (TAG)	G to T	Tv nonsense
25	N-T		PCA/Gleason 3, T3	8/279	Gly (GGG) → Gly (GGT)	G to T	Tv silent
26	N-T		PCA/Gleason 3, T3	5/151	Pro (CCC) → Pro (CCG)	C to G	Tv silent
Non-castrated group (*n*=13)							
6	N-T		PCA/Gleason 2, T3	7/235	Asp (AAC) → Thr (ACC)	A to C	Tv missense
	N-T		PCA/Gleason 3, T3	8/296	His (CAC) → Asn (AAC)	C to A	Tv missense
9	N-T		PCA/Gleason 2, T3	7/243	Cys (TGC) → Tyr (TAC)	G to A	Ts missense
16	N-T	1	HGPIN	5/145	Gly (GGC) → Asp (GAC)	C to G	Ts silent
	N-T	1	HGPIN	5/128	Pro (CCT) → Leu (CTT)	C to T	Ts missense
17	N-T	2	HGPIN	8/285	Glu (GAG) → Stop (TAG)	G to A	Ts nonsense
	N-T	1	HGPIN	8/291	Lys (AAG) → Thr (ACG)	A to C	Tv missense
19	N-T		PCA/Gleason 3, T3	5/164	Lys (AAG) → Lys (AAA)	G to A	Ts silent
				5/154	Gly (GGC) → Asp (GAC)	G to A	Ts missense
	N-T		PCA/Gleason 3, T3	5/172	Val (GTT) → Ala (GCT)	T to C	Ts missense
	N-T	0	HGPIN	5/142	Pro (CCT) → Leu (CTT)	C to T	Ts missense
	N-T	1	HGPIN	7/248	Arg (CGG) → Arg (AGG)	C to A	Tv silent
20	N-T		PCA/Gleason 5,T3	5/151	Pro (CCC) → Pro (CCG)	C to G	Tv silent
				5/167	Gln (CAG) → Gln (CAA)	G to A	Ts silent
	N-T		PCA/Gleason 5, T3	5/172	Val (GTT) → Ala (GCT)	T to C	Ts missense
	N-T	1	HGPIN	5/145	Leu (CTG) → Leu (TTG)	C to T	Ts silent
	N-T	0	HGPIN	5/141	Leu (CTG) → Leu (TTG)	G to A	Ts missense

PIN, prostatic intraepithelial neoplasia; PCA, prostatic carcinoma; Ts, transition; Tv, transversion; T, transition zone; N-T, non-transition zone.
a) Mutation at CpG sites.

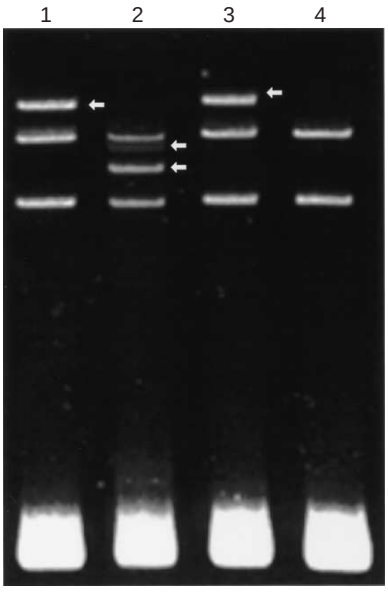

Fig. 3. PCR-single-strand conformation polymorphism (SSCP) analysis of *p53* mutation. Nonradioactive SSCP analysis of exon 7. Aberrant migration patterns (arrow) were seen in lane 1 (case 6, PCA), lane 2 (case 3, PCA), and lane 3 (case 19, PIN). Wild-type SSCP bands are shown in lane 4 (case 19, benign prostatic hypertrophy).

Table II. Summary of *p53* Mutations in PCA and PIN

	Mutation frequency (%)							
	Case	Lesion	Stage		Zone		Distance from PCA	
			T2	T3	Transition	Non-transition	≤2 mm	>2 mm
PCA	8/27 (29.6)	11/55 (20.0)	1/22 (4.5) *a*)	10/33 (30.3) *a*)	1/25 (4.0)	10/30 (33.3) *a*)	—	—
PIN	6/27 (22.2)	13/111 (11.7)	4/33 (12.1)	9/78 (11.5)	2/36 (5.6)	11/75 (14.7)	11/46 (24.0) *a*)	2/65 (3.0)

a) $P<0.05$.

Table III. Frequency of *p53* Mutations in Non-castrated Group

	Mutation frequency (%)							
	Case	Lesion	Stage		Zone		Distance from PCA	
			T2	T3	Transition	Non-transition	≤2 mm	>2 mm
PCA	4/13 (30.8)	7/24 (29.2)	0/5 (0)	7/19 (36.8)	0/5 (0)	7/19 (36.8)	—	—
PIN	4/13 (30.8)	8/56 (14.3)	2/14 (14.3)	6/42 (14.3)	0/20 (0) *a*)	8/36 (22.2) *a*)	9/31 (29.0) *b*)	0/25 (0) *b*)

a) $P<0.05$.
b) $P<0.01$.

the current series, there is a difference in mutation frequency of PCA lesions by stage of disease: one of 22 lesions (4.5%) in T2 and 10 of 33 lesions (30.3%) in T3 showed mutations. This suggest that *p53* gene mutations might be involved in the progression of PCA. Berner *et al.* (1995) reported that C-to-G transversion at codon 273 was frequently found in PCA.[23] They suggested that this might be a mutational hotspot in the progression of PCA. Although exon 5 was the commonest site for mutations in our previous[15] and the current series, there were no mutational hotspots. Mutation at the CpG site was found in one case (case 21). G-to-A transition and C-to-G transversion substitution were most common in our series. In 8 of 12 cases with PCA and/or PIN lesions, *p53* mutations had at least one mutation that changed an amino acid, which might have provided the selection pressure for expansion.

Multiplicity of PCA with various grades of histologic differentiation is common. A previous study using the fluorescence *in situ* hybridization (FISH) technique showed allelic loss of 8p in PIN and PCA lesions,[24] and suggested a common "genetic history" for these proliferations. Further, a FISH study revealed a diverse pattern of allelic loss of 8p[24] and *c-myc* overexpression[25, 26] from tumor to tumor or gland to gland. Konishi *et al*. reported different patterns of *p53* alterations among multifocal lesions of PCA.[27] Recently we reported the multiclonal development of prostatic precancerous lesions, based on the analysis of microdissected specimens.[15] In cases 19 and 20 of the current series, the direct sequencing of the PCR-SSCP products from 4 independent foci, 2 PIN and 2 PCA, showed different patterns of *p53* mutations, indicating each focus to be derived from a different clone. The presence of different clones in the same prostatic lesions was also shown in another 5 cases (cases 6, 16 17, 18 and 21). Multiclonality of prostatic precancerous and cancerous lesions is not surprising in the light of multistep carcinogenesis. The presence of precancerous lesions on the verge of becoming cancer should be taken into account when treating patients with PCA. Our recent study [8] showed that the HGPIN, like PCA, is sensitive to androgen deprivation therapy.

The non-transition zone is known to be the predominant site for PCA and HGPIN.[28, 29] Coexistence of PCA and HGPIN lesions in the non-transition zone was found in approximately 75% of PCA cases,[8] supporting a precancerous nature of HGPIN. In our previous study,[15] mutations of *p53* gene in the HGPIN and PCA were analyzed in the prostatectomy specimens as a whole but not by zone

(i.e., the non-transition and transition zone). The current study revealed that the frequency of *p53* mutations in PCA lesions was significantly higher in the non-transition than in the transition zone. As for HGPIN, *p53* mutations in the non-transition zone were significantly more frequent than those in the transition zone among non-castrated cases. These findings suggest that *p53* gene mutations play a role in the development of precancerous and cancerous lesions in the non-transition zone, but not in the transition zone.

The distance between the PCA and HGPIN was reported to be frequently within 2 mm.[5, 30] Bostwick and Brawer reported that the frequency of appearance of HGPIN was increased in cases with PCA compared to those without PCA.[1] In our previous study[8] close association (distance within 2 mm) of HGPIN with PCA was more frequently found in the non-transition zone (63% of lesions) than in the transition zone (38% of lesions). These findings provided support for the precancerous nature of HGPIN, especially in the non-transition zone. Indeed, the frequency rate of *p53* mutations in HGPIN lesions close to PCA (24% of lesions in the total and 29% in the non-castrated cases) was significantly higher than that in lesions distant from PCA (>2 mm) (3% in the total and none in the non-castrated cases). These findings further support the precancerous nature of the HGPIN close to PCA.

Our previous study suggested a relationship between HGPIN and PCA with regard to *p53* mutation and multiclonal development of prostatic precancerous lesions.[15] The current study using the laser capture microdissection method clearly showed a significant involvement of *p53* gene mutations in the development of HGPIN and PCA in the non-transition zone, and suggests a precancerous nature of HGPIN located close to PCA.

(Received April 24, 2000/Revised June 15, 2000/Accepted June 22, 2000)

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