Expression of the *prolactin-inducible protein* (*PIP/GCDFP15*) gene in benign epithelium and adenocarcinoma of the prostate

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Prolactin-inducible protein (PIP), also known as gross cystic disease fluid protein 15, is a predominant secretory protein in various body fluids, including saliva, milk and seminal plasma. Immunohistochemistry of this protein has been exploited as a clinical marker for breast cancer and Paget's disease. This study comparatively examined PIP expression in normal prostate tissues and in adenocarcinomas of the prostate. Quantitative real-time RT-PCR revealed low-level presence (6%) of PIP mRNA in normal prostate tissue in comparison with the seminal vesicle. Indirect immunostaining with monoclonal antibody 3E7 displayed a positive sign for benign epithelium in 8 cases (29.6%) among 27 normal specimens; however, the incidence significantly increased to 56.1% (37/66) in instances involving primary prostate carcinoma tissues of different types. Quantitative RT-PCR also demonstrated that PIP transcript levels in carcinoma regions were significantly higher than corresponding levels in benign regions. These findings conclusively showed that benign prostate epithelium expresses PIP at low levels; in contrast, PIP is over-expressed in carcinomas of the prostate. (Cancer Sci 2004; 95: 491–495)

Gross cystic disease fluid protein 15 (GCDFP15) is a 15- to 17-kDa glycoprotein isolated from human breast cystic fluid.[1] This protein is also known as prolactin-inducible protein (PIP), which has been isolated separately from breast cancer cells of the T47D cell line[2] (PIP is used preferentially in accordance with IUPAC nomenclature guidelines). PIP is commonly present in various body fluids, such as saliva, tears, milk and sweat, secreted by exocrine organs, which include the salivary, lacrimal and mammary glands. The PIP molecule found in seminal plasma encoded by the same gene is designated independently as gp17 consequent to posttranslational modification, which differs from that of PIP/GCDFP15.[3,4] PIP expression in mucosal cells is characteristically dependent on reproductive hormones, namely prolactin and androgen, hormone-responding elements of which, i.e. the signal transducer activator of transcription-5 and androgen receptor element, reside within the 5′-promotor region spanning approximately 1.4 kb.[5,6] In terms of physiological function, PIP/gp17 is capable of suppressing T-cell apoptosis in the reproductive system via high-affinity interaction with CD4, which appears to compete with HIV on lymphocytes.[7]

Immunohistochemical analysis of this protein has been utilized as a clinical marker of apocrine cells for pathological examination. In particular, an extensive investigation by Wick *et al*. demonstrated that PIP expression can be detected by immunohistochemical assay in up to 76% of breast cancers; as a result, the presence of PIP, as well as estrogen receptor, in metastatic tumors of unknown origin is widely accepted as an indicator of breast malignancies.[8–10] However, the specificity to breast cancer remains controversial.[4]

In the human male genital tract, glandular epithelium of the seminal vesicle is the primary source of PIP, but PIP production has not been detected in other organs.[1,11–13] On the other hand, approximately 10% of prostate cancer tissues immunohistochemically react with PIP.[8,14] Hall *et al*. reported that PIP is co-expressed with prostate-specific antigen (PSA), a major prostate cancer marker, in androgen receptor-positive breast tumors.[15] Moreover, a number of DNA sequences that coincide with that of PIP cDNA are derived from the prostate, as seen in human expressed sequence tag (EST) libraries. These data suggest that *PIP* gene expression possibly occurs in a wider variety of tissues than is currently believed. PIP production in normal prostate was re-evaluated in the present study. Furthermore, immunoreactivity and PIP transcripts were comparatively analyzed with primary prostate carcinoma tissues.

Materials and Methods

Materials. A total of 93 archival sample tissues (46, 14 and 33 from needle biopsy, surgical operation and autopsy, respectively) were available from pathological profiles at the Yamagata University Faculty of Medicine. Specimens included 27 normal prostate (mean age, 65.8 years) and 66 primary prostate adenocarcinoma (mean age, 71.2 years) tissues. Tissues were fixed in 10% neutral buffered formalin, followed by paraffin embedding. Diagnosis of carcinoma was based on judgment of hematoxylin-eosin stained slides, in which malignancy was determined by Gleason's score.[16] A subset of 11 tissues obtained during surgery ($n=10$) and autopsy ($n=1$) was also stored at −80°C until required for RNA preparation. Seminal vesicle tissue was available from a surgical case. Patients and a family member provided informed consent for the experimental use of these samples.

Monoclonal antibodies (mAbs). A peptide oligomer VDVIRELGIC, corresponding to amino acid positions 114 to 123 in the human PIP sequence, was synthesized at Sawady (Tokyo); keyhole-limpet hemocyanin (KLH) was used as a carrier protein. To select a unique sequence, 10 consecutive amino acids were compared with the available genome database by employing FASTA at the DDBJ web site (http://www.ddbj.nig.ac.jp). BALB/c mice were immunized by repeated injections with the antigen in Freund's adjuvant (Difco, Detroit, MI). After fusion of spleen cells with P3U1 myeloma cells in the presence of polyethylene glycol 4000 (Wako, Osaka), the hybridoma cells were cloned by limiting dilution; positives were screened by enzyme-linked immunosorbent assay (ELISA) and immunoblotting using the culture supernatants. Ascites fluid was obtained by intraperitoneal injection of 10^7 cells into pristine-

primed mice. Anti-human PSA mAb Ab-1, raised in mice, was purchased from Oncogene Research Products (San Diego, CA).

Immunoblotting. A DNA segment was amplified from PIP cDNA using restriction site-incorporating oligonucleotide primers, 5′-ctcgaattcGTTCTCTGCCTGCAGTTGGG-3′ and 5′-gagaagcttTTATTCTACCTTTAGGATTTC-3′.[11] The fragment was inserted into an expression vector, pMAL-2 (New England Biolabs, Beverly, MA), containing the *maltose-binding protein* (*MBP*) gene. Competent *Escherishia coli* XL-1 cells were transfected with the plasmid, and a single colony was selected and cultured in LB medium supplemented with 0.5 m*M* isopropyl thiogalactopyranoside at 37°C. As a control, the plasmid vector without the insert was used for transfection. Prior to immunoblotting, cell pellets were lysed with a bacterial protein extraction reagent, B-PER (Pierce, Rockford, IL). Following denaturation by boiling for 3 min in the presence of SDS and mercaptoethanol, aliquots of 3 μl of the bacterial extract and seminal plasma were electrophoresed in 10–20% gradient polyacrylamide gels; subsequently, samples were transferred onto PVDF membrane sheets. The strips were incubated with the cloned antibody at 37°C for 1 h, followed by incubation with peroxidase-conjugated anti-mouse IgG (Dako, Copenhagen, Denmark). The ECL-Plus western blotting detection system (Amersham Pharmacia, Buckinghamshire, UK) was utilized for detection of the interaction.

Reverse transcription-PCR (RT-PCR). Total RNA was extracted from tissues of the prostate and seminal vesicle employing spin columns from an RNeasy Mini Kit (Qiagen, Hilden, Germany). The initial strand of cDNA was synthesized from 500 ng of RNA extracts in a volume of 20 μl using AMV reverse transcriptase XL (TaKaRa, Otsu) priming with random 9-mers at 42°C for 10 min. Subsequently, PCR amplification proceeded in a volume of 20 μl, which included 1 μl of the cDNA transcripts, with HotStar *Taq* DNA Polymerase (Qiagen), through 35 rounds of the following temperature cycle: 94°C for 20 s, 56°C for 20 s and 72°C for 20 s. Three pairs of oligonucleotide primers were designed (forward, 5′-GGCCAACAAAGCT-CAGGACAAC-3′ and reverse, 5′-TGCACCTTGTAGAGG-GATGCTG-3′), (forward, 5′-TGCGAGAAGCATTCCCAAC-3′ and reverse, 5′-GGAAGCTGTGGCTGACCTG-3′) and (forward, 5′-TGTTGCCATCAATGACCCCTTC-3′ and reverse, 5′-AGCATCGCCCCACTTGATTTTG-3′) for the *PIP*, *PSA* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) genes, respectively. Forward and reverse primers were selected from separate exons to avoid amplification of any genomic DNA. Sizes of these PCR products ranged from 172 to 198 bp. Amplified products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide.

Quantitative real-time RT-PCR. Total RNA was isolated from 20-μm cryotome sections of stored frozen tissues. All cDNAs were prepared by reverse transcription as described above. A volume of 1 μl of the first strand DNA was employed for quantitation of specific cDNAs with the Light Cycler system (Roche Diagnostics, Mannheim, Germany). Real-time quantitative RT-PCR reactions were performed with the QuantiTect SYBR green PCR kit (Qiagen) as recommended by the manufacturer. The same oligonucleotide primers used for RT-PCR were utilized in the reactions. Amplification consisted of an initial 15-min preheating step at 95°C, followed by 40 cycles of 94°C for 15 s, 56°C for 25 s and 72°C for 20 s. Sensitivity of reactions and the amplification of contaminant products, indiscriminately detected by the SYBR green chemistry, were evaluated via amplification of serial dilutions of 4.2×10^8 DNA copies (1:10, 1:100, $1:10^3$, $1:10^4$ and $1:10^5$) and a blank without cDNA. The relationship between the fractional cycle and the fluorescence threshold was confirmed ($r=-1$). Results were analyzed with Light Cycler software Ver.3.5 (Roche Diagnostics).

Immunohistochemistry. Paraffin sections (4 μm), placed on ProbeOn Plus slide-glass (Fisher Scientific, Pittsburg, PA), were deparaffinized; subsequently, samples were rehydrated in xylene and graded alcohols. Endogenous peroxidase activity was blocked by incubation in methanol containing 0.3% hydrogen peroxide for 10 min at room temperature. Specimens were incubated with ascites of mAb 3E7 to PIP or mAb Ab-1 to PSA at a dilution of 1:100 at 37°C for 30 min. The aforementioned steps were conducted with the Envision kit (Dako), with diaminobenzidine as the substrate. Following hematoxylin counterstaining, specimens were mounted. Rinsing was performed three times in each step with 10 m*M* phosphate-buffered saline for 5 min. Specific antibodies were replaced with non-immune mouse serum (Dako) at the identical dilution in order to generate negative controls. Results were assessed semi-quantitatively
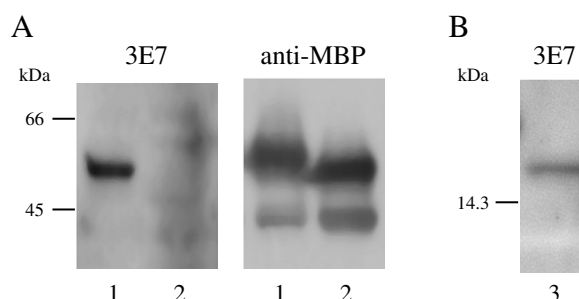

Fig. 2. Specificity of mAb 3E7 to human PIP molecules evaluated by immunoblotting. (A) Recombinant fusion molecules were expressed in *E. coli* cells transfected with PIP cDNA-inserted pMAL plasmid vector. Transblotted strips were incubated with mAb 3E7 (left) and anti-MBP (right). Lanes 1, fusion molecules of PIP and MBP; 2, non-fused MBP. (B) Immunostaining of seminal plasma with mAb 3E7. Lane 3, seminal plasma (3 μl) from a healthy volunteer.
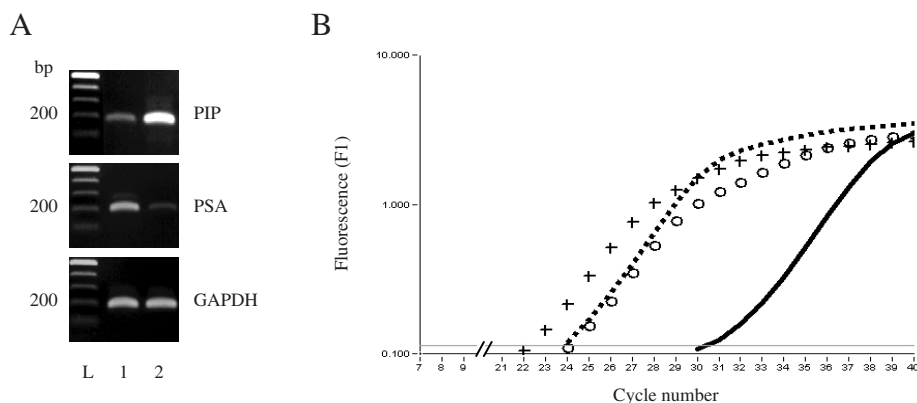

Fig. 1. Agarose gel electrophoresis of RT-PCR products (A) and amplification chart of real-time RT-PCR from extracts of normal tissues (B). (A) Amplification of the PIP, PSA and GAPDH transcripts: lanes 1, prostate; 2, seminal vesicle. (B) PIP and GAPDH amplifications from extracts of the prostate and seminal vesicle; PIP cDNA in prostate (━), PIP cDNA in seminal vesicle (○), GAPDH cDNA in prostate (▪▪) and GAPDH cDNA in seminal vesicle (+).

by two researchers according to the percentage of stained cells among 200 cells as follows: less than 1%, negative; 1%–10%, weakly positive; 10%–50%, moderately positive; and greater than 50%, strongly positive.

Statistical analyses and database search. Comparison of immunohistochemical findings between normal and carcinoma tissues and between PIP and PSA expression was assessed by use of the χ^2 test. Differences in PIP transcript number of normal and carcinoma tissues were calculated by the parametric t test. A search of a database containing deposited ESTs was conducted at the DDBJ site (http://www.ddbj.nig.ac.jp).

Results

Expression analysis based on RT-PCR in the normal prostate. Production of PIP/gp17 in the prostate has not been documented; consequently, expression in two normal samples of seminal vesicle and prostate was comparatively examined by RT-PCR using oligonucleotide primers designed from the cDNA sequence. A 172 bp PIP transcript was evident in the extract from prostate; however, the signal intensity was weaker than that of the seminal vesicle (Fig. 1A). Subsequently, quantitative real-time RT-PCR was conducted to extract samples from prostate and seminal vesicle, in which the transcript of the gene encoding GAPDH served as an internal control of cDNA synthesis and PCR (Fig. 1B). The PIP transcript ratio of prostate to seminal vesicle was calculated to be 0.06.

Immunohistochemistry in normal prostate tissue. Based on a general procedure regarding utilization of the synthetic peptide, of which the sequence corresponds to that of human PIP, a mAb, designated as 3E7, was obtained in mice; its subclass was determined to be IgG1 by ELISA. Immunoblotting with recombinant fusion molecules and seminal plasma revealed specific reactivity of 3E7 to PIP (Fig. 2), indicating that this mAb rec-ognized a sequence-dependent epitope on PIP. Furthermore, 3E7 reacted with formalin-fixed paraffin-embedded sections including submandibular gland and seminal vesicle.

Immunohistochemical assay of normal prostate tissue using this mAb 3E7 afforded eight positive signals among 27 samples characterized by diffuse granular staining in luminal and basal cells of the epithelium (Fig. 3A). The same sample employed in the former molecular analysis did not react immunohistochemically with 3E7, which might be attributable to the different sensitivities of the two procedures.[10] For the positive control, immunohistochemical analysis involving mAb to PSA was performed under identical conditions. Positive staining was observed in benign epithelium with the exception of a single specimen.

Immunohistochemistry to prostate carcinomas. Enhanced PIP expression has been demonstrated in breast cancer.[10, 13] Therefore, immunoreactivity to mAb 3E7 in prostate carcinoma tissues was examined. In a total of 66 samples, 37 (56.1%) were positive for PIP based on pancytoplasmic and granular staining (Fig. 3, B to D). The incidence was significantly higher than that of the normal tissues ($P<0.05$), accompanied by increased intensity, as summarized in Table 1. This observation indicates that up-regulated expression of the *PIP* gene may occur during malignant transformation. These specimens consisted of a variety of tissue types, with Gleason scores ranging from 5 to 9. However, staining did not correlate with the histological scores (Fig. 4); furthermore, staining was also unrelated to histological differentiation. PSA immunostaining exhibited statistically significant co-expression with PIP in prostate cancer (Table 2).

Comparative quantitation of PIP transcript. Transcripts were analyzed in subsets consisting of 15 and 11 specimens of normal regions and carcinomatous ones, respectively, derived from 11 dissected tissues by means of quantitative real-time RT-PCR in order to evaluate the enhanced expression of the *PIP* gene in
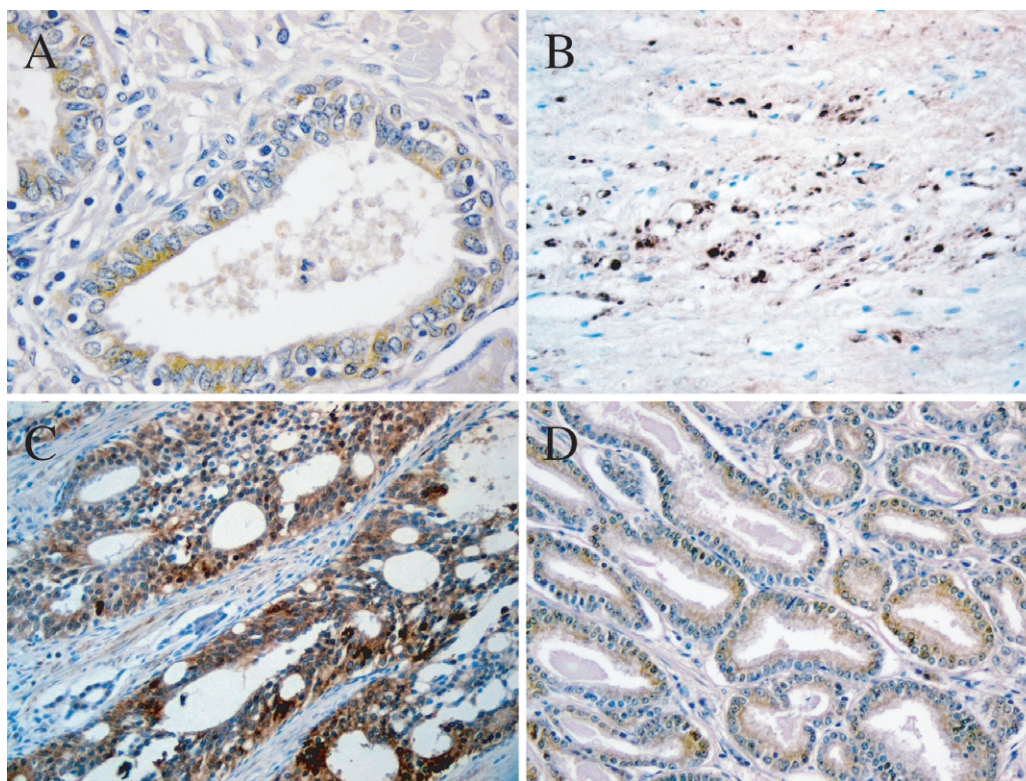

Fig. 3. Immunohistochemisry with mAb 3E7 to PIP. (A) Normal glandular epithelium of the prostate, (B) poorly differentiated adenocarcinoma (Gleason grade 5), (C) moderately differentiated adenocarcinoma (Gleason grade 4), (D) well-differentiated adenocarcinoma (Gleason grade 3). Original magnification is ×50 or ×100.

Table 1. Immunoreactivity to PIP and PSA in normal tissues and carcinomas of the prostate

	Total	Immunoreactivity				Positive (%)
		−	+	++	+++	
PIP (prolactin-inducible protein)						
Normal	27	19	4	3	1	29.6
Carcinoma	66	29	7	16	14	56.1[1]
PSA (prostate-specific antigen)						
Normal	27	1	1	9	16	96.3
Carcinoma	66	10	0	24	32	84.8

[1] $\chi^2=5.36$, $P<0.05$.

greater detail (Fig. 5). The mean value of PIP expression in tumors exceeded that of benign tissues. The difference was statistically significant ($P<0.002$).

Discussion

In the present series of investigations, PIP transcripts from normal prostate epithelium were detected by RT-PCR and immunohistochemical techniques, despite the belief that the seminal vesicle is the exclusive producer of PIP in the male reproductive tract. Quantitative real-time RT-PCR gave a positive result even in an instance involving content as low as 6%, compared to that of the seminal vesicle. An EST database at DDBJ includes lists of partial cDNA clusters that enable identification of tissues in which a specific gene is transcribed. In June 2003, the database contained 79 deposited EST sequences encoding PIP. Twenty-one of these ESTs were derived from the prostate. This database analysis supported the present results regarding PIP expression in the prostate. As additional proteins produced predominantly by the seminal vesicle, the paralogous genes of semenogelin 1 and 2 are well known. Transcription of the semenogelin genes has been documented in the seminal vesicle as well as in the prostate and vas deferens, in a manner similar to PIP.[17]

PIP/gp17 is the major component of seminal plasma that is present in the range of 0.2 to 1 mg/ml, but the physiological importance of production in the prostate is obscure.[3, 11] PIP exhibits binding ability to CD4, which is thought to suppress HIV infection.[7] Further, PIP inhibits the growth of *Streptococcus* in culture by interaction with microorganisms.[18] The evidence suggests local immune system involvement as a function. In another respect, Bergamo *et al.* reported that human PIP/gp17 also interacts with the spermatozoa cell surface via CD4, which is speculated to be related with capacitation ability.[19] Moreover, mouse seminal vesicle autoantigen (SVA), another member of the *PIP* gene family, is abundant in seminal plasma. This SVA exerts an inhibitory effect on spermatozoa mobility via interaction with cell membrane phospholipids.[20, 21] Thus, it is supposed that PIP molecules play andrological roles in relation to motility of spermatozoa.

In terms of immunohistochemical analysis, mAb 3E7, which reacts with all species of PIP molecules through recognition of a sequence-dependent epitope, was employed. In addition, a second mAb to PIP/GCDFP15, D6 (Signet Lab., Dedham, MA), which is commercially available, was examined. However, mAb D6 was less effective in the prostate; moreover, D6 did not correlate significantly with 3E7. The D6 antibody does not react with antigen denatured in the presence of detergents such as SDS, which may imply recognition of a conformational epitope. This may be related to the fact that D6 reacts with a portion of heterogeneous PIP molecules generated by distinct post-transcriptional modifications, including glycosylation.[4, 22]

The incidence and intensity of positive specimens were significantly increased in adenocarcinomas as detected by immu-

Table 2. Co-expression of PIP and PSA in carcinoma of the prostate

		PIP	
		Negative	Positive
PSA	Negative	9	1
	Positive	20	36

$\chi^2=6.33$, $P<0.02$.

nohistochemistry using mAb 3E7, and the level of PIP transcripts was elevated in the analysis by quantitative real-time RT-PCR. These results, which conclusively showed that over-expression of the *PIP* gene occurred in primary prostate carci-
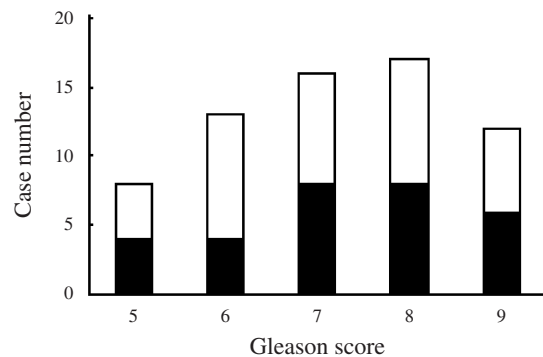

Fig. 4. Gleason scores of prostate carcinomas and incidence of PIP-positive specimens by immunohistochemistry with mAb 3E7. Filled and empty bars correspond to numbers of specimens displaying positive and negative signs, respectively.
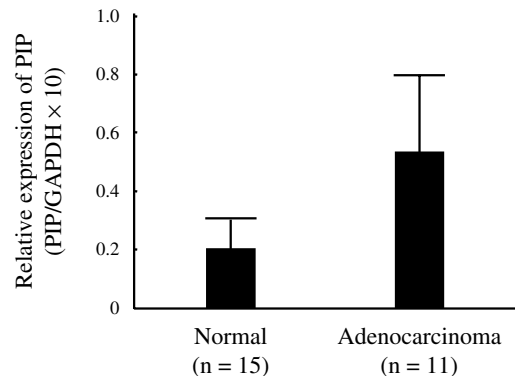

Fig. 5. Comparison of PIP expression relative to GAPDH between normal prostate tissues and prostate carcinomas by quantitative real-time RT-PCR. Mean value and SD are indicated. Higher PIP expression in carcinoma was statistically significant relative to that in normal tissues ($P<0.002$).

nomas, were consistent with the findings of Clark *et al.*, who reported that transcription was enhanced in primary and metastatic breast cancer tissues.[10] Possible explanations of PIP over-expression in prostate carcinoma are discussed below.

The *PIP* gene is located on human chromosome 7q34. This telomeric region has been identified as a common fragile site (CFS) in the human genome, which is characterized by rearrangements, and in which breakpoints fall within chromosomes. In human chromosome 7q, FRG7H has been identified as a typical CFS at 7q32. For instance, loss of heterozygosity occurs in this vicinity of the *MET* oncogene in breast and prostate cancers.[23, 24] The region of 7q34 is involved in the adjacent FRG7I, at which frequent unstable alterations are also observed; furthermore, this PIP locus is targeted in cancer cells. Recent reports demonstrated that this gene is subject to a variety of phenomena, such as frequent nucleotide substitutions and small circular DNA fragment formation.[25, 26] In particular, Ciullo *et al.* showed by fluorescence *in situ* hybridization that the *PIP* gene is inversely duplicated in T47D culture cells by the hypothetical mechanism of 'breakage-fusion-bridge,' which leads to over-expression.[27] Jones *et al.* performed genome-wide

analysis of breast carcinomas by comparative genomic hybridization; their findings revealed the presence of frequent gains in DNA copy number at the telomeric region of 7q.[28] Therefore, it seems likely that instability events accompanied by increased gene copy number may be responsible for enhanced detection of *PIP* gene expression in prostate carcinomas.

It is noteworthy that PIP production in exocrine organs is dependent on androgens, including testosterone. Androgen receptor is over-expressed in prostate carcinomas, and in particular, hormone-refractory tumors often develop androgen receptor gene amplification in their genome.[29, 30] PIP over-expression is possibly involved in the signaling pathway regulated by the androgen receptor.[13]

In conclusion, PIP, which is distributed in benign epithelium of the prostate, is enhanced in carcinomas; as a result, PIP immunostaining may make a useful contribution as a diagnostic index of malignant transformation in the prostate. Moreover, PIP has been employed as a clinical marker for breast carcinoma as well as for mammary and extra-mammary Paget's disease. However, further re-evaluation of its tissue distribution is necessary.

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