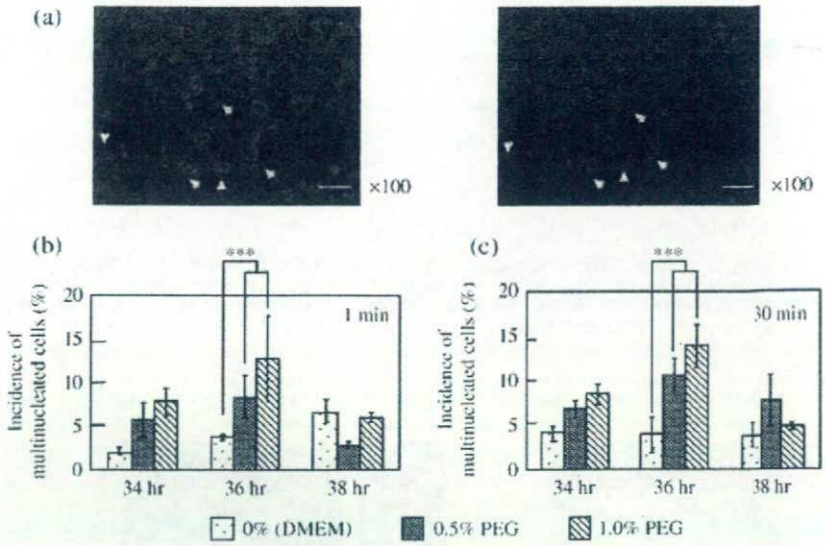

Fig. 1. Polyethylene glycol (PEG) induces multinucleated cells. (a) Following the PEG treatment protocol, PC-3 cells were treated with 0.5% PEG for 30 min. Cells were stained with propidium iodide (PI) (red). The left panel indicates the morphological features of the PEG-treated cells under microscopic observation with a green filter. The right panel indicates the nuclear staining of cells with PI. Both panels are the same magnification. The arrows indicate typical multinucleated cells. Scale bars = 50 μm. (b,c) Incidences of multinucleated cells in a total of 500 cells treated with PEG for (b) 1 min and (c) 30 min are shown. Values are the mean of fold ± SD. ***Differences of cell number between PEG treatment (0.5 and 1.0%) and the Dulbecco's modified Eagle's medium (DMEM)-treated control ($P < 0.001$).

Effects of PEG on in vivo tumor growth. The effect of PEG on PC-3 xenograft tumor growth was evaluated under two different conditions *in vivo*. With one of these, following the PEG treatment protocol, PC-3 cells (5×10^6 cells) were pretreated with DMEM or 0.5% PEG for 30 min, and then treated cells were implanted subcutaneously into the backs of BALB/c-nu/nu male mice (CLEA, Tokyo, Japan). Alternatively, PC-3 cells (5×10^6) were first implanted subcutaneously into the back of each mouse, and then at day 7 after implantation, 200-μL aliquots of PBS alone or 0.5% PEG solution in PBS were injected directly into the tumors using a 25-gauge injection needle (Terumo Corporation, Tokyo, Japan). Thereafter, tumors were measured twice a week throughout the experimental period of 19 days, and tumor volume was calculated using the following formula: (length [mm]) × (width [mm])^2 × 0.52. In these experiments, a total of 16 nude mice was randomly separated into four groups (4 × 4) receiving: DMEM treatment; 0.5% PEG treatment; intratumor PBS injection; and intratumor 0.5% PEG injection. PC-3 cells were implanted at two sites for each mouse. Animal experimental protocols were approved by the Committee for Ethics in Animal Experimentation, and the experiments were conducted in accordance with the guidelines for Animal Experiments of the National Cancer Center (Tokyo, Japan).

Histopathological analysis and detection of apoptosis in PC-3 xenografts. PC-3 xenografts were extirpated at 36–38 h after the last injection of PBS or 0.5% PEG for fixation in 10% neutralized formalin and embedding in paraffin blocks. Serial sections were prepared at 3.5-μm thickness, stained with hematoxylin and eosin (H&E), and subjected to histopathological analysis by a trained pathologist (M. W.). Apoptotic cells were detected using the TdT-mediated dUTP-biotin nick end-labeling (TUNEL) method,[32] which was carried out *in situ* using the DeadEnd Colorimetric TUNEL System (Promega) following the manufacturer's protocol.

Statistical analysis. All statistical analyses were carried out with Wilcoxon signed-ranks test using KaleidaGraph software (Synergy Software, Reading, PA, USA). Differences were considered significant when the *P*-value was less than 0.05.

Results

Induction of multinucleated PC-3 cells by PEG treatment *in vitro*. With the PEG treatment protocol, higher numbers of multinucleated cells were observed after PEG treatment, using 0.5–1.0% PEG (Fig. 1a). The incidence of multinucleated cells peaked at 36 h, being 8.4 and 13% with 0.5 and 1.0% PEG, respectively (Fig. 1b). The incidence only slightly increased with a longer exposure to PEG for 30 min (Fig. 1c).

Effects of PEG on PC-3 cell growth *in vitro*. In order to exclude possible toxic effects of PEG on PC-3 cells, we cultured PC-3 cells under two different culture conditions. When PC-3 cells were propagated under ordinary culture plates, and PEG was added into the media, no significant effect on cell growth was observed up to 72 h in the presence of 0.5 and 1.0% PEG, compared with the non-PEG treated control (Fig. 2a). However, significant differences were observed in cell growth between PEG-treated and DMEM-treated control cells when PC-3 cells were treated with 0.5% PEG (Fig. 2b) and 1.0% PEG (Fig. 2c) at a high cell density for 5–30 min, as in the PEG treatment protocol. A decrease in cell numbers became most prominent at around 36 h after PEG treatment (Fig. 2b,c).

Biochemical analyses for apoptosis. We observed nuclear condensation in multinucleated PC-3 cells on staining with Hoechst 33258 (Fig. 3a), suggesting induction of apoptosis.[33] Although DNA ladder formation was not observed in PEG-treated PC-3 cells, DNA samples extracted from the cells treated with PEG were substantially degraded compared to the DMEM-treated control (data not shown). Furthermore, PARP-1 cleavage, another characteristic feature of apoptosis,[34] was demonstrated in a PEG dose-dependent manner (Fig. 3b). The ratio of cleaved PARP-1 was increased by 14–52% in PEG-treated PC-3 cells when compared to the DMEM-treated control cells, although the increase was not so drastic (Fig. 3c).

Quantification of apoptosis by FCM. FCM analysis by double staining with Annexin-V (horizontal axis) and PI (vertical axis) demonstrated substantial induction of apoptosis, as depicted in Figure 4a. The incidence of cells in the early apoptotic phase (Annexin-V$^+$, PI$^-$) was 16.3%, and that in late apoptotic phase (Annexin-V$^+$, PI$^+$) was 9.2% after treatment with 0.5% PEG (Fig. 4a right). An approximate 10-fold increase was observed in the percentage of Annexin-V-positive cells (25.5%) with PEG treatment compared to 2.8% for the DMEM-treated control (Fig. 4a left). Furthermore, multinucleated cells demonstrated positive staining for Annexin-V (arrows in Fig. 4b).

In order to clarify whether apoptosis was induced mainly in multinucleated cells, we extracted FCM data sets of only large cells (Fig. 5a), and then replotted the PI signals of each cell against the Annexin-V signals using the above data (Fig. 5b). In Figure 5a, the number of cells in the large-cell fraction (R1), the cut-off value for which was set arbitrarily, increased almost three-fold (8.52 vs 24.26% among 10 000 cells analyzed) after

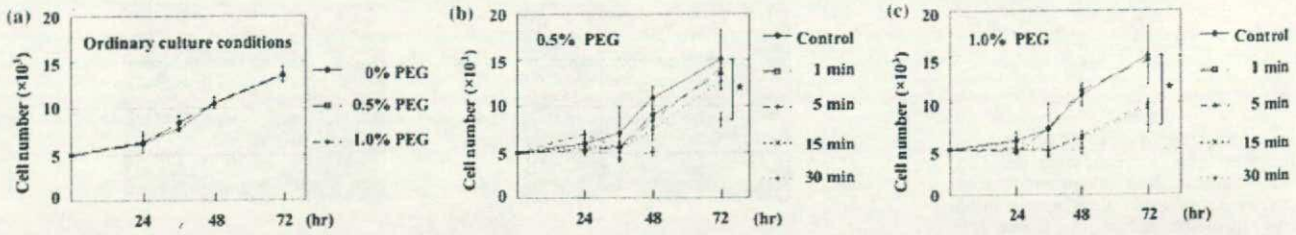

Fig. 2. Polyethylene glycol (PEG) suppresses cell growth at a high cell density. (a) After PEG solution was added into culture media, viable PC-3 cells were counted. (b,c) Following the PEG treatment protocol, PC-3 cells were treated with (b) 0.5% or (c) 1.0% PEG for 1, 5, 15, and 30 min, and viable cells were counted. Values are the mean of fold ± SD. (b) Differences of cell number between 0.5% PEG (30 min) and the Dulbecco's modified Eagle's medium (DMEM)-treated control, and (c) those between 1.0% PEG (30 min) and DMEM-treated control were significant. *$P < 0.05$.

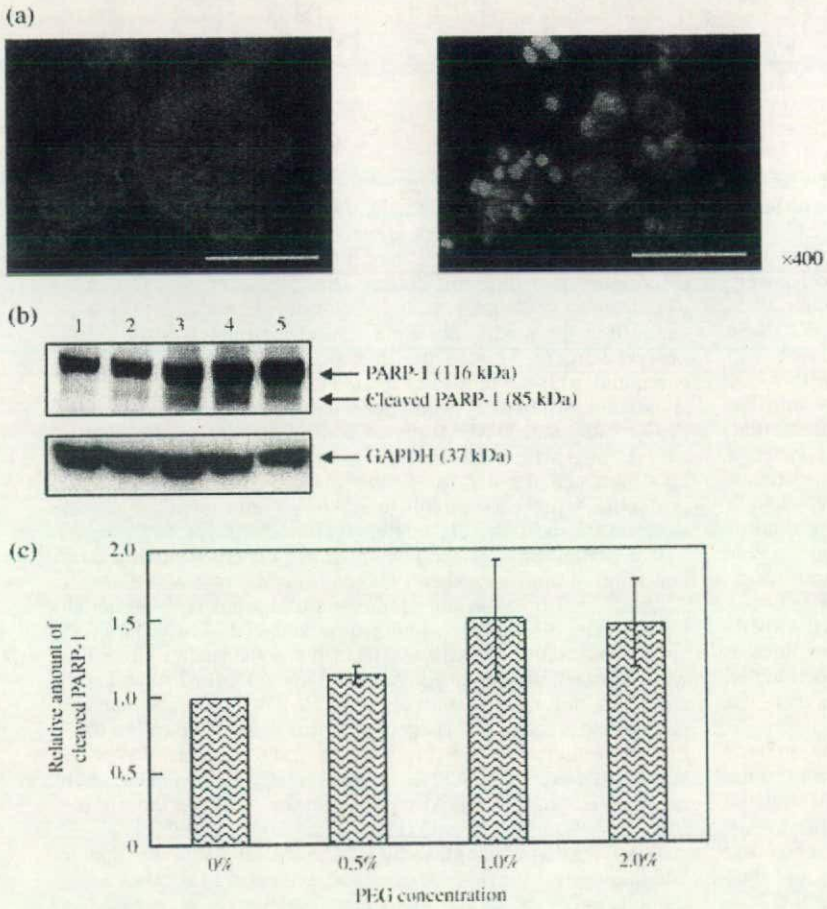

Fig. 3. DNA condensation and poly(ADP-ribose) polymerase (PARP)-1 cleavage. (a) PC-3 cells were treated with 1.0% polyethylene glycol (PEG) for 30 min following the PEG treatment protocol. Cells were stained with Hoechst 33258 (blue), as detailed in Materials and Methods. The left panel indicates the typical morphological feature of multinucleated cell (arrows). The right panel indicates the nuclear condensation of multinucleated cells. Both panels are the same magnification. Scale bars = 50 μm. (b) PARP-cleavage. Whole-cell lysates after treatment with Dulbecco's modified Eagle's medium (DMEM), PEG, or etoposide were prepared, and full-length (uncleaved) PARP-1 (116 kDa), cleaved PARP-1 (85 kDa), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (37 kDa) were immunoblotted. Lane 1, DMEM; lane 2, 0.5% PEG; lane 3, 1.0% PEG; lane 4, 2.0% PEG; lane 5, etoposide (positive control). (c) Quantification of the cleaved PARP-1 protein by western blot analysis. The density of both noncleaved and cleaved PARP-1 and of the background levels were measured, and the ratios of cleaved PARP-1 against the total PARP-1 were calculated as follows: (density of cleaved PARP-1) – (background level)/(density of total PARP-1) – (background level).

0.5% PEG treatment. In Figure 5b, among the cells in the R1 fraction, 292 and 1571 cells in DMEM-treated control and 0.5% PEG-treated group respectively, showed positive for Annexin V. Namely, an approximate five-fold increase in apoptotic cells was observed within the large-cell fraction of PEG-treated cells compared to DMEM-treated cells. In particular, a more than 20-fold increase was observed in the number of cells in early apoptotic phase (25 vs 656 cells). Similar results were obtained with various cut-off values for R1 to include 3.0% and 5.5% of total populations in DMEM-treated cells (data not shown).

No evidence of mitotic catastrophe in PEG-treated cells. By FCM analysis, no marked changes in cell cycle profile were apparent after PEG treatment (Fig. 6a). Although the number of cells in the sub-G_1 fraction was slightly increased by PEG treatment, cells in the G_1, S, G_2/M, and > G_2/M fractions were not changed significantly (Fig. 6b). We also noted that the number of cells in > G_2/M fractions decreased substantially between 34 and 36 h after PEG treatment, as depicted in Supplemental Fig. 1.

We then extracted aneuploid-cell and large-cell populations from the > G_2/M fraction using the FCM data set, as detailed in Supplemental Fig. 2A, and the corresponding number of cells in Supplemental Fig. 2A was counted between 34 and 36 h (Supplemental Fig. 2B). The number of aneuploid cells in the > G_2/M fraction did not show a significant change between 34 and 36 h after 0.5 and 1.0% PEG treatment. However, an approximate 20% increase in the numbers of large cells in the > G_2/M fraction was observed 34 h after PEG treatment, which substantially decreased at 36 h (Supplemental Fig. 2B). These results further

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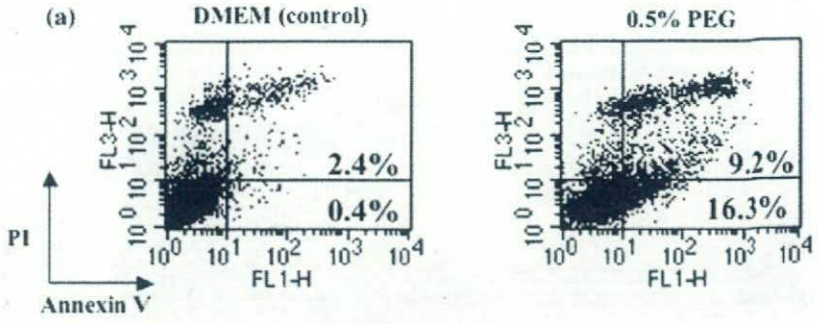

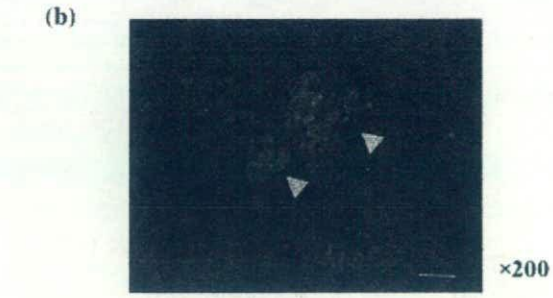

Fig. 4. Quantification of apoptosis using flow cytometry (FCM). (a) PC-3 cells were treated with Dulbecco's modified Eagle's medium (DMEM) alone (left) or 0.5% polyethylene glycol (PEG) (right) for 1 min following the PEG treatment protocol. After double-staining with Annexin-V and propidium iodide (PI), apoptotic cells were analyzed by FCM. (b) Multinucleated giant cells (arrow) visualized by staining with Annexin-V (green) and PI (red). Scale bar = 50 μm.

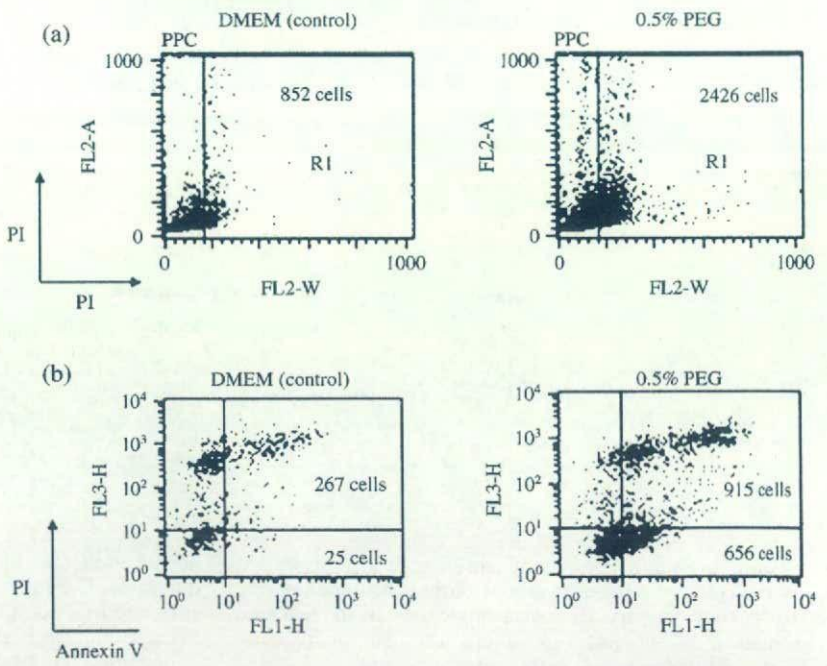

Fig. 5. Induction of apoptosis in large cells induced by polyethylene glycol (PEG) treatment. (a) From the data sets presented in Figure 4a, large cell populations were examined. (b) On the data sets presented in (a), the large cells gating the R1 region were replotted by propidium iodide (PI) signals against Annexin-V.

support the view that reduction of cell numbers by PEG treatment occurs in large-cell fractions, possibly by apoptosis, but not by mitotic catastrophe in aneuploid cells.

Induction of apoptosis by PEG in PC-3 xenografts. Following the PEG treatment protocol, PC-3 cells were pretreated with DMEM or 0.5% PEG and then implanted into mice. At day 7, the tumor volume of the PEG-treated group was significantly reduced compared to the DMEM-treated group. An approximate 50% reduction in tumor volume was observed with PEG treatment (Fig. 7a). To further confirm the growth-suppressive effect of PEG in an *in vivo* setting, we conducted the following experiment. Non-treated PC-3 cells were implanted into mice, and PBS or 0.5% PEG solution was injected into palpable tumors from day 7, twice a week (Fig. 7b top). The volumes of PC-3 xenografts increased approximately eight-fold from day 7 to day 25 in the PBS-injected group (Fig. 7b bottom). In contrast, substantial suppression of tumor growth was detected after injecting 0.5% PEG solution directly into tumors. The average sizes of tumors in PBS- and 0.5% PEG-injected groups at day 25 were 1344 ± 292 and 812 ± 215 mm^3, respectively. Namely, tumor volumes were reduced approximately 30% in the PEG-injected group compared with those in the PBS group at

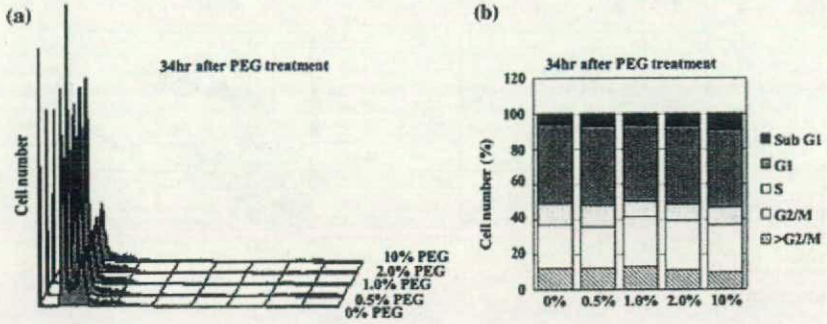

Fig. 6. Cell cycle analysis. (a) Cell cycle profiles after polyethylene glycol (PEG) treatment at 34 h are demonstrated. (b) From the data sets presented in (a), the cell population of each fraction (sub-G_1, G_1, S, G_2/M, and > G_2/M fraction) in the cell cycle is shown.

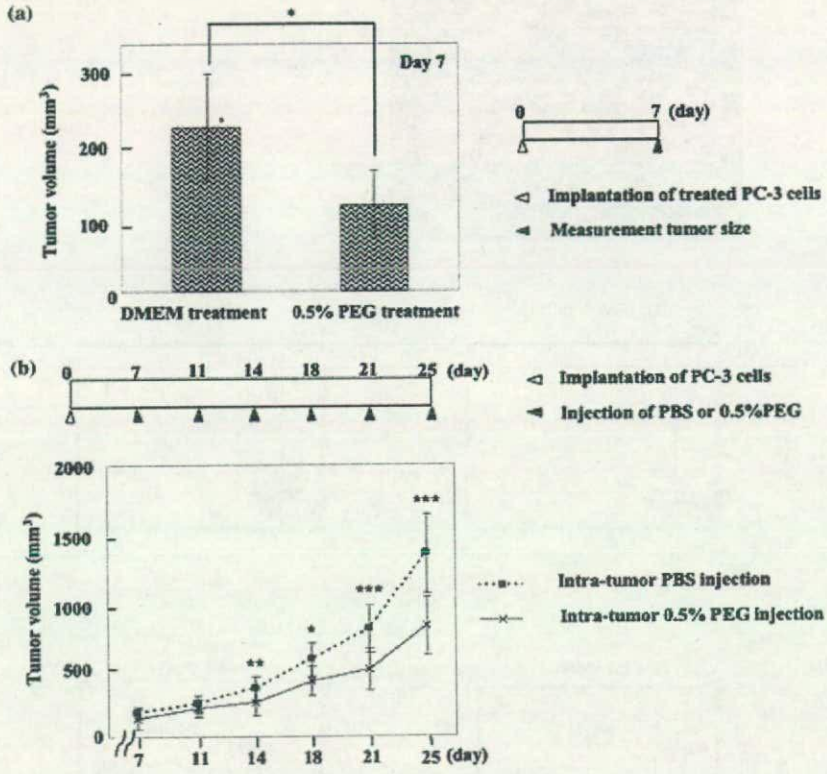

Fig. 7. Growth-suppressive effects of 0.5% polyethylene glycol (PEG) on PC-3 xenografts in nude mice. (a) After PC-3 cells were treated with Dulbecco's modified Eagle's medium (DMEM) or 0.5% PEG for 30 min, the cells were implanted into the backs of nude mice at day 0. Tumor volumes were measured at day 7. (b) Experimental protocols. PC-3 cells were implanted into backs of nude mice at day 0, and then 200 μL aliquots of phosphate-buffered saline (PBS) or 0.5% PEG was injected into palpable tumors twice a week during 19 days (top). Growth curves on PC-3 xenografts injected with PBS (■) or 0.5% PEG (X) are demonstrated (bottom). Values are the mean of fold ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

day 25 (Fig. 7b bottom). No significant change in bodyweight was observed between the two groups during the experimental period (data not shown). Histopathological analysis revealed the presence of vacant spaces in tumors at the site of injection (Fig. 8a–d). Multinucleated cells were frequently observed surrounding these spaces in PEG-injected groups, and multinucleated cells were observed in approximately 20% of the total TUNEL-positive cells. In contrast, few TUNEL-positive cells were detected in PBS-injected tumors, although necrotic lesions were similarly observed. Multinucleated cells were rarely apparent in PBS-injected tumors (Fig. 8c,e).

Discussion

The present study demonstrated the antitumor effects of PEG via multinucleated cell formation, presumably though the induction of cell fusion. This is in line with the recent report by Roy *et al*. that PEG 800 induces apoptosis in two human colonic adenocarcinoma cells, HT29 and CaCo-2, at concentrations of 3.6–4.8%.[35] Although they briefly described PEG-induced cell fusion to be a possible mechanism, they considered this unlikely because a much higher concentration of PEG, around 30–50%, is generally required to induce cell fusion.[35]

In the present study, we were indeed able to demonstrate that low concentrations of PEG could induce apoptosis via multinucleated cell formation. More surprisingly, nuclear condensation was evident in a fraction of the multinucleated cells. As it has been reported that the cell-fusion process occurs within 1–2 h, mitotic catastrophe should be considered as a causative event for the induction of cell death by PEG.[36] However, the number of aneuploid cells in the > G_2/M fraction did not show a significant change during this time, and the number of large multinucleated cells decreased between 34 and 36 h. Therefore, an abrupt decrease in cell numbers after PEG treatment of PC-3 cells *in vitro* is reasonably explained by the induction of apoptosis in multinucleated cells, although clumped cells with aneuploid

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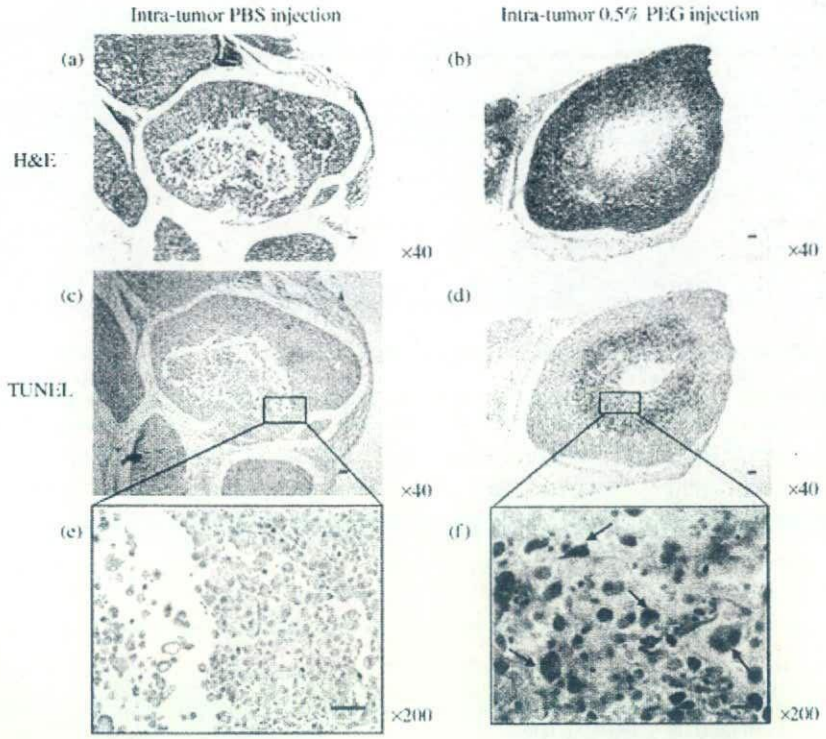

Fig. 8. Induction of multinucleated cells and apoptosis by polyethylene glycol (PEG) injection in PC-3 xenografts. Tumors with similar sizes were selected and allotted randomly into two groups. Aliquots (200 μL) of phosphate-buffered saline (PBS) or 0.5% PEG were injected directly into each group of tumors at days 7, 11, 14, 18, 21, and 25. Representative histological features of tumors in experimental groups of (a,c,e) PBS injection and (b,d,f) PEG injection are presented. The tumor sizes at day 7 before the PBS and 0.5% PEG injections were 180 ± 42 and 140 ± 70 mm^3, respectively. (a,b) Hematoxylin and eosin (H&E) staining. (c–f) TdT-mediated dUTP-biotin nick end-labeling (TUNEL) immunohistochemical staining. (e,f) Magnified images of (c) and (d), respectively. The arrows indicate typical multinucleated cells positive for TUNEL staining. Scale bars = 50 μm.

characteristics may also be a part of the large-cell fraction. Further work is needed to substantiate this possibility, but the positive staining for Annexin-V and PI and PARP-1 cleavage provide some support for this hypothesis.

Taking the results together, it is plausible that multinucleated cell formation is a trigger and causative event for the induction of apoptosis by PEG. Although an increase in osmotic pressure by PEG has been considered to be important for its cytostatic or apoptotic effects,[35] it is unlikely that osmotic pressure played a role with the low concentrations of PEG used here. Indeed, when PC-3 cells were cultured in the presence of PEG under ordinary culture conditions, we did not detect apparent changes in cell-growth properties for up to 72 h (Fig. 2a). This negates the possible implication of osmotic pressure in the induction of apoptosis by the concentrations of PEG used in the present conditions. Another possibility to be considered is that cell-membrane damage caused by PEG led to the induction of apoptosis. It is thought that the cell membrane could be damaged by injection of cells with PEG particles through microcylinders in *in vivo* models. Further study is warranted to corroborate or refute this point. However, the induction of nuclear condensation, positive staining for Annexin-V in multinucleated cells, and PARP-1 cleavage by PEG treatment all point to a significant role for apoptosis in the growth-suppressive effects of PEG. Although the mechanism of induction of apoptosis by PEG remains unclear, we clearly demonstrated that PEG exhibited a suppressive effect on tumor growth *in vivo* and induced apoptosis, possibly via the cell-fusion mechanism.

Lastly, we should note that PC-3 prostate cancer cells were used as a model system. The therapies for prostate cancer, for example hormone therapy or radiation therapy, have some drawbacks. Radioactive material such as iodine-125 using brachytherapy may cause chromosomal aberrations.[37] Occurrence of androgen-independent prostate cancers because of frequent or continuous application of hormone therapy is a very serious problem at present. Once cancer becomes androgen independent, metastatic lesions manifest aggressively within 12–18 months, and the average patient survival time is only 2–3 years.[38] Although chemotherapies have been adapted for androgen-independent aggressive cancer cases, the tumor-suppressive effects brought about by those therapies are not satisfactory.[39] Therefore, for cases with localized non-invasive lesions, urologists generally choose radical prostatectomy.[40] At the same time, however, there are a substantial number of cases in which radical therapy cannot be conducted because of the poor compliance of patients and life-threatening side effects.[41,42]

In the present study, we clearly demonstrated that PEG is able to induce apoptosis in PC-3 cells in an autonomous cell-fusion manner, with efficient suppression of tumor growth *in vivo*. Based on our observations, we propose that direct intratumoral injection of PEG could be a promising therapeutic approach for androgen-independent prostate cancers. Clearly, if PEG is injected in clinical trials, it may induce cell fusion for normal prostate cells, and unwanted side effects may appear. However, even if PEG induces multinucleated cells between normal and tumor cells, it could be effective for diffuse types of prostate cancer. In addition, direct intratumoral injection of PEG could also assist hormone therapy and radiation therapy. Although several problems remain to be solved, the potential of PEG as a novel, non-invasive and non-toxic therapeutic agent clearly warrants further attention.

Acknowledgments

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Supplementary materials

The following supplementary material is available for this article:

Fig. S1. The cell number of > G_2/M fractions decreased between 34 and 36 h. From the data sets presented in Figure 6a, the cell number of > G_2/M fractions was counted between 24 and 38 h using flow cytometry.

Fig. S2. Disappearance of large cells between 34 and 36 h. (A) From flow cytometry data sets, the aneuploid cell (A) and large-cell (L) populations were extracted, as described elsewhere. (B) From the data sets presented in (A), each cell population was monitored between 34 and 36 h.

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Hormone receptor-related gene polymorphisms and prostate cancer risk in North Indian population

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Abstract The purpose of this study was to analyse the frequency and type of mutations in the coding region of androgen receptor (AR) and to determine the role of polymorphisms in the intron 1 of *ERα*, exon 5 of *ERβ*, intron 7 of progesterone, exon 7 of the aromatase (*CYP19*) and exon 9 of *VDR* genes in the risk of prostate cancer. PCR-RFLP analysis of all above the genes was on 100 prostate cancer patients and an equal number of matching controls. The study also included PCR-SSCP analyses of exons 2–8 of *AR* gene. The genotype containing −/− allele of *ERα* gene was statistically significant for the risk of prostate cancer pose (OR, 2.70; 95% CI, 1.08–6.70, $P = 0.032$) Rr genotype of *ERβ* gene also have a higher risk (OR, 1.65; 95% CI, 0.52–5.23) for prostate cancer. The *Cys* allele of *CYP19* gene was also associated with statistically significant increased risk of prostate cancer (OR; 2.28, 95% CI, 1.20–4.35, $P = 0.012$). tt genotype of codon 352 of *VDR* gene showed an OR of 0.43 for (95% CI, 0.13–1.39) and an OR for Tt genotype was 0.65 (95% CI, 0.36–1.16). Taken together, the results showed that in North Indian population, *ERα* and *CYP19* genes may be playing a role in the risk of prostate cancer.

Keywords Prostate carcinoma · AR · ERs · PR · CYP19 · VDR gene polymorphisms

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Introduction

Epidemiological evidences have suggested that steroid hormones are involved as initiators or promoters in prostate carcinogenesis and that hormonal factors may be associated with the risk of prostate cancer [1, 2]. Androgens are required for normal male sexual development, growth, differentiation, maintenance of secondary sexual characteristics and spermatogenesis. Androgen receptor (AR) is a member of the nuclear receptor family that includes receptors for steroid and thyroid hormones, vitamin D_3 and retinoic acids, and numerous orphan receptors for which no ligands are known [3]. The AR was first described in 1969 [4] and cloned in 1988 [5]. The gene is located on the X-chromosome at Xq11–12 and spans a length of approximately 90-kb of DNA and contains eight exons [6]. The AR has been proposed as a candidate gene for several cancers (breast, prostate, uterine endometrium, colon and esophagus) [7].

Similarly, ER is expressed in a wide variety of cell types in addition to estrogen-responsive tissues such as mammary epithelium, endometrium, breast and bone. Two different *ER*s, i.e., *ERα* [8] and *ERβ* [9] have been

characterized. The human *ERα* encoding gene located on chromosome 6q24–27 consists of eight exons, seven introns, about 140 kb in length, two promoter regions and five functional domains designated A/B-F [10] result in two transcripts that differ only in the 5′ region. The protein itself has 595 amino acids with molecular weight of 66,182 Da. The *ERβ* gene is located on chromosome 14q23–24.1 [11] and consists of eight exons [12]. The DNA binding domain of *ERβ* is highly homologous to that of *ERα*, implying that both *ERα* and *ERβ* share the same DNA response element.

The human progesterone receptor gene (*HPR*) is located on chromosome 11q22–23 and its product belongs to the steroid thyroid-retinoic acid receptor superfamily of transcription factors [13]. The action of progesterone is mediated via the progesterone receptor (PR). Progesterone binds to PR and the activated PR protein translocates to the cell nucleus under the control of hinge region signals. PR–PR homodimers are formed and these interact with hormone response elements of PR-responsive genes to regulate their transcription. Thus, the cellular responses to progesterone are influenced by both PR protein level and activity of progesterone hormone levels.

Aromatase P450 is present in the endoplasmic reticulum of estrogen-reproducing cells in which it is expressed. The effects of the resulting estrogens are mediated through the estrogen receptor. The prostate $P450_{arom}$ is encoded by the *CYP19* gene localized on chromosome 15p21.1 [14] which spans at least 52 kb and contains nine coding exons beginning with exon II and an untranslated first exon (5′-termini) whose transcripts differ from one another in a tissue-specific fashion [15]. The prostate is influenced by estrogen from peripheral sources as well as through aromatase activity in its stroma [16]. Data from a prospective study suggest that the low levels of estradiol may be a risk factor for prostate cancer [17]. There is an evidence of elevated levels of aromatase activity and mRNA expression in stroma cells in prostate cancer [18], as well as increasing the evidence of cross talk between estrogens and androgens in regulating gene expression in the prostate [19]. Moreover, circulating estrogens can compete with androgens for binding to sex hormone-binding globulin, and it is generally assumed that sex hormone-binding globulin synthesis is regulated by and is a reflection of the estrogen/androgen balance [20].

Vitamin D and its metabolites are best known for their action in calcium and bone metabolism. However, epidemiological studies have suggested that an increased prostate cancer risk is associated with decreased production of vitamin D. The effects of vitamin D and its analogous are mediated by vitamin D receptor (VDR), a member of the steroid/thyroid/retinoic acid receptor superfamily, which functions as a ligand-dependent transcription factor [21]. The gene encoding VDR located on chromosome 12cen-q [22], contains 14 exons, and spans more than 75 kb of genomic DNA [23]. Bid et al. [24] determined the distribution of VDR gene (Fok-I, Taq-I and Apa-I) polymorphisms using a PCRRFLP analysis in unrelated normal healthy individuals from a north Indian population. Their results suggested that the frequency and distribution of the polymorphisms in India are substantially different from these in other populations and ethnic groups.

The genes mentioned above reveal polymorphisms and there are reports of some of them to be the risk factors for prostate cancer in populations other than India. It is, therefore, pertinent to study such polymorphisms in multiethnic Indian populations to look for genetic risk factors for prostate cancer.

Materials and methods

Patients and controls

A study group consisting of 100 prostate cancer patients were collected from the Department of Urology, Government Medical College and Hospital, Chandigarh, India. The control group consisted of 100 male healthy controls that visited Postgraduate Institute of Medical Science and Research (PGIMER) for minor treatments. All subjects were from the same ethnic group. They were asked about their age, cigarette smoking and alcohol drinking habits. Prostate cancer patients were all histologically confirmed and characterized in terms of their clinical staging (Tumour-Node-Metastasis system) and grade (Gleason score). Nine per cent (9.0%) of the patients had well differentiated, 54% moderate-differentiated and 37% poorly differentiated adenocarcinoma. Regarding clinical staging, 22% were in stage A, 15% in stage B, 23% in stage C and 40% in stage D. The informed consent was obtained from both of patients and controls.

DNA extraction

Genomic DNA of all cases and controls was isolated from either blood or frozen tissues using proteinase K digestion followed by phenol/chloroform extraction method [25] and stored at −20°C.

PCR-SSCP

The analysis of AR was based on the PCR amplification with primers given in Table 1. Amplification consisted of an initial denaturation step for 5 min at 95°C, 35 cycles of denaturation at 95°C and primer extension at 72°C for

Table 1 PCR primers for AR exons 2–8

Primers pairs	Sequence of primers	Fragment size (bp)	*AT_m (°C)
AR-2F	5′-GCC ATT CAG TGA CAT GTG TTG CAT TGG	266	60
AR-2R	5′-AAG GTT AGT GTC TCT CTC TGG AAG GT-3′		
AR-3F	5′-AAC TCA TTA TCA GGT CTA TCA ACT C-3′	243	60
AR-3R	5′-AAA ATC TGG TCT AAA GAG AGA CTA GA-3′		
AR-4F	5′-GCA TTG TGT GTT TTT GAC CAC TGA TG-3′	385	60
AR-4R	5′-GAT CCC CCT TAT CTC ATG CTC CC-3′		
AR-5F	5′-CAA CCC GTC AGT ACC CAG ACT GAC CA-3′	285	60
AR-5R	5′-AGC TTC ACT GTC ACC CCA TCA CCA TC-3′		
AR-6F	5′-CTC TGG GCT TAT TGG TAA ACT TCC-3′	294	57
AR-6R	5′-GTC CAG GAG CTG GCT TTT CCC TA-3′		
AR-7F	5′-GCT TTG TCT AAT GCT CCT TCG TGG-3′	271	60
AR-7R	5′-CTC TAT CAG GCT GTT CTC CCT GAT-3′		
AR-8F	5′-GAG GCC ACC TCC TTG TCA ACC CTG-3′	304	57
AR-8R	5′-CCA AGG CAC TGC AGA GGA GTA GTG GC-3′		

* AT_m is annealing temperature of each primer

Table 2 Distribution of allelic variants in prostate cancer patients and controls

Genotype	Cases	Controls	OR (95% CI)	*P*-value	OR (95% CI)[a]	*P*-value
+/+	28 (28%)	42 (42%)	1.00		1.00	
+/−	54 (54%)	48 (48%)	1.68 (0.91–3.12)	0.096	1.72 (0.92–3.20)	0.084
−/−	18 (18%)	10 (10%)	2.70 (1.08–6.70)	0.032	2.74 (1.10–6.84)	0.030
(+/−) + (−/−)	72 (72%)	38 (38%)	1.94 (0.86–3.23)	0.028	1.97 (1.09–3.57)	0.024
RR	92 (92%)	95 (95%)	1.00		1.00	
Rr	8 (8.0%)	5 (5.0%)	1.65 (0.52–5.23)	0.394	1.74 (0.54–5.55)	0.350
A1/A1	84 (84%)	90 (90%)	1.00		1.00	
A1/A2	16 (16%)	10 (10%)	1.71 (0.73–3.98)	0.211	1.74 (0.74–4.05)	0.200
CC	59 (59%)	73 (73%)	1.00		1.00	
CT	37 (37%)	20 (20%)	2.28 (1.20–4.35)	0.012	2.35 (1.23–4.49)	0.010
TT	4 (4.0%)	7 (7%)	0.70 (0.19–2.53)	0.594	0.70 (0.19–2.54)	0.596
CT + TT	41 (41%)	27 (27%)	1.87 (1.03–3.40)	0.038	1.91 (1.05–3.49)	0.033
TT	55 (55%)	43 (43%)	1.00		1.00	
Tt	40 (40%)	48 (48%)	0.65 (0.36–1.16)	0.147	0.66 (0.37–1.18)	0.164
tt	5 (5.0%)	9 (9.0%)	0.43 (0.13–1.39)	0.160	0.41 (0.12–1.33)	0.138
Tt + tt	45 (45%)	55 (55 %)	0.61 (0.35–1.07)	0.090	0.62 (0.35–1.08)	0.095

OR, odds ratio; CI, confidence interval

$P < 0.05$ was considered statistically significant

[a] ORs adjusted for age

1 min each. These were followed by a final elongation step for 5 min at 72°C (Figs. 1–3).

DNA sequencing

PCR products that showed normal and shifted SSCP patterns were purified directly from the PCR reaction using QIA quick PCR purification Kit (Qiagen, Germany). Their sequences were determined by cycle sequencing using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) on an automated DNA-sequencer ABI PRISM 310 (Applied Biosystems/ Perkin-Elmer).

PCR-RFLP

Estrogen receptor genotyping

The analysis of *ERα* and detection of the *PvuII* restriction endonuclease site in intron 1 was facilitated by PCR amplification of a region spanning the site with primers,

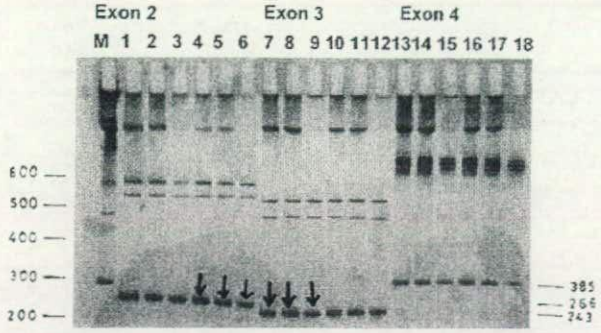

Fig. 1 DNA Silver Stained SSCP Gene Gel Excel 12.5/24 (AR gene exons 2–4). Lane M, 100 bp Marker (Invitrogen); lanes 1–6, exon 2 (266 bp); lanes 7–12, exon 3 (243 bp); lanes 13–18, exon4 (385 bp)

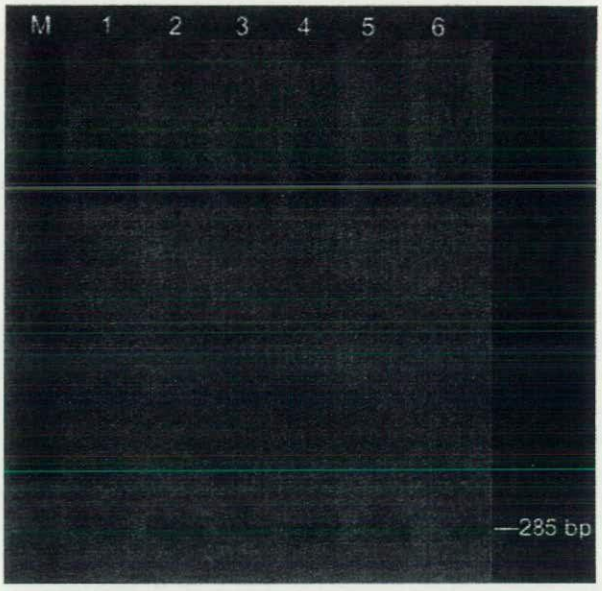

Fig. 2 PCR-SSCP analysis of *AR* gene exon 5. DNA Silver Stained SSCP Gene Gel Excel 12.5/24. Lane M, 100-bp Marker (Invitrogen); lanes 1–6, exon 5 (285-bp)

5′-ATCCAGGGTTATGTGGCAATGAC-3′ and 5′-ACCC TGGCGTCGATTATCTGA-3′. Amplification consisted of an initial 5 min denaturation at 95°C, 30 cycles of denaturation at 95°C, annealing at 57°C and elongation at 72°C for 1 min each. These were followed by a final elongation step for 5 min at 72°C. Then 10 µl aliquots of PCR products were digested with 5 units of *PvuII* restriction enzyme (New England, Biolabs, USA) followed by incubation at 37°C for 2 h. The presence of the *PvuII* restriction sites is indicated by a (+), whereas the absence of the site is denoted by a (−) (Fig. 4).

The analysis of *ERβ* was based on the PCR amplification with the primers 5′-CAGGCTTTGTGGAGCTCAG-3′ and 5′-ACCTGTCCAGAACAAGATCT-3′. Amplification consisted of an initial denaturation step for 10 min at 95°C, 30 cycle of denaturation at 95°C, annealing at 62°C and primer extension at 72°C for 1 min each. These were followed by a final elongation step for 5 min at 72°C. An aliquot 10 µl of PCR products was digested overnight at 37°C with 5 units of *RsaI* (Takara Shuzou, Kyoto, Japan) restriction endonuclease. Presence of the silent mutation in codon 328 resulted in cleavage of 144 bp PCR product with *Rsa*I restriction endonuclease into two fragments of 110 and 34 bp (Fig. 5).

Progesterone genotyping

The analysis of PROGINS was based on the PCR amplification of a fragment encompassing the 306 bp insertion polymorphism in intron 7 using the primers 5′-GCCTCTAAAATGAAAGGCAGAAAGC-3′ and 5′-GCGCGTATTTTCTTGCTAAATGTCTG-3′. Amplification was carried out for 30 cycles with denaturation at 94°C, annealing at 60°C and extension at 72°C for 1 min each. An initial denaturation step was carried out for 3 min at 94°C and a final extension for 5 at 72°C min. The A1 allele of HPR was defined as the absence of the insertion. A1 allele appeared as a 175-bp fragment and the A2 allele PROGINS appeared as a 481-bp fragment (Fig. 6).

Aromatase genotyping

PCR amplification of the coding and flanking sequence of exon 7 of the aromatase gene was carried out using the primers 5′-CGCTAGATGTCTAAACTGAG-3′ and 5′-CATATGTGGCATGGGAATTA-3′. Thermal cycling consisted of an initial 5 min denaturation step at 95°C, followed by 35 cycles of denaturation at 95°C, annealing at 51°C, elongation at 72°C for 1 min each, and a final elongation for 5 min at 72°C. Then 10 µl aliquots of the PCR products were digested at 37°C for 2 h with 5 units of *Sfa*NI (New England, Biolabs, USA) restriction enzyme. The C to T substitution in exon 7, resulting in a single amino acid substitution from Arg to Cys at codon 264, creates a recognition site for the SfaNI restriction enzyme (Fig. 8).

VDR genotyping

A 740-bp fragment encompassing the *TaqI* polymorphism site in exon 9 of *VDR* gene was amplified using primers 5′-CAGAGCATGGACAGGGAGCAA-3′ and 5′-GCAACTC CTCATGGCTGAGGTCTC-3′. Thirty-five cycles were performed using Taq polymerase with denaturation at 95°C, annealing at 64°C and extension at 72°C for 1 min each. Thereafter, 10 µl of the PCR product was subjected to restriction digestion with *TaqI* (Takara Shuzou, Kyoto, Japan) and incubated at 65°C for 3 h, and run directly on 2.0% agarose gel followed by staining with ethidium

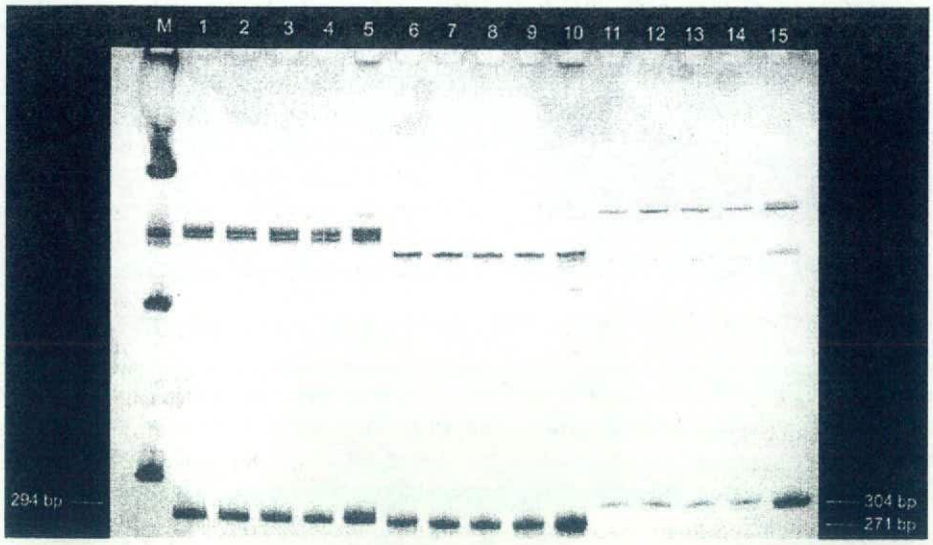

Fig. 3 DNA silver stained SSCP Gene Gel Excel 12.5/24 (AR gene exons 6–8). Lane M, 100 bp Marker (Invitrogen); lanes 1–5, exon 6 (294 bp); lanes 6–10, exon 7 (271 bp); lanes 11–15, exon 8 (304 bp)

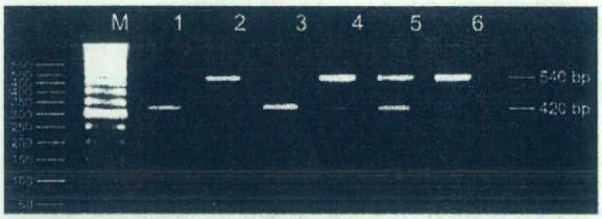

Fig. 4 RFLP-analysis of *ERa* gene using Pvull restriction enzyme. Lane M, 50-bp Marker (Invitrogen); lanes 1 and 3, +/+ (420-bp); lanes 2, 6, −/− (540-bp); lanes 4 and 5, +/− (420, 540-bp)

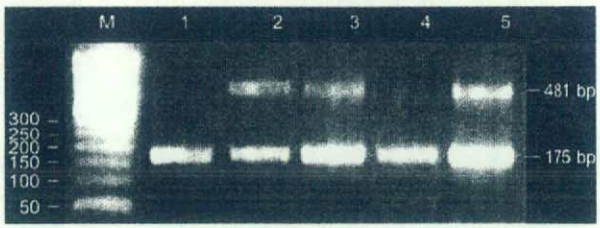

Fig. 6 PCR amplification of *PR* gene intron 7. Lane M, 50-bp Marker (Invitrogen); lanes 1, 4, A1/A1 (175-bp); lanes 2, 3, 5, A1/A2 (175, 481-bp)

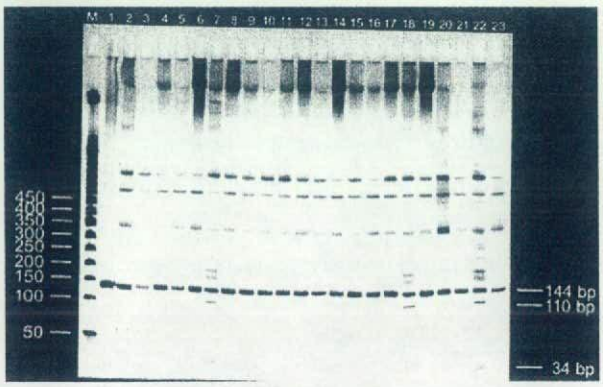

Fig. 5 RFLP analysis of ER-β gene using Rsa-I restriction enzyme. Lane M, 50 bp Marker (Invitrogen); lanes 1–6, 8–17, 19–21, 23, RR (144 bp); lane 7, 18, 22, Rr (34, 110, 144 bp)

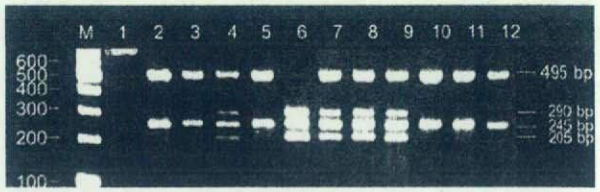

Fig. 7 RFLP-analysis of VDR gene using TaqI restriction enzyme. Lanes M and 1, 100-bp marker (Invitrogen); lanes 2, 3, 5, 10–12, PCR product (740-bp); lane 6, TT (245, 495-bp), lanes 4 tt (205, 245, 290-bp); lanes 7–9 Tt (205, 245, 290, 495-bp)

bromide. The presence of C to T change at the third position of codon 352 for isoleucine in exon 9 is associated with the loss of a *TaqI* restriction site. The resulting alleles were designated T (TaqI site absent; two fragments of 495 and 245 bp) or t (TaqI site present; three fragments of 290, 245 and 205 bp). Individuals were classified as TT, Tt or tt (capital letter indicates the absence of restriction site). Amplification was performed in a final volume of 50 µl containing 40 ng of genomic DNA, 1 unit of Taq polymerase, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate, 0.3 µM of each primer and 5 µl of ten times PCR buffer (500 mM KCl and 200 mM Tris–HCl) and water to a total volume of 50 µl (Fig. 7).

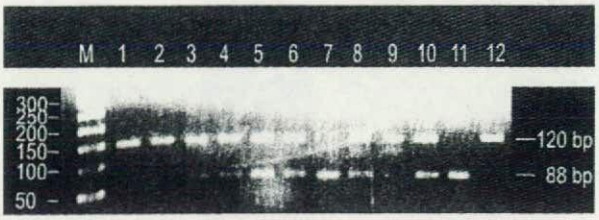

Fig. 8 RFLP-analysis of *CYP19* gene using SfaNI restriction enzyme. Lane M, 50-bp Marker (Invitrogen); lanes 1, 2, 9, 12, CC (120-bp); lanes 7 and 11, TT (88-bp); lanes 3–6, 8 and 10, CT (120, 88-bp)

Statistical analysis

In order to examine the association between the genotypes and the development of prostate cancer, we calculated odds ratio (OR) and 95% confidence interval (CI). The ORs were adjusted for age using multiple logistic regression analysis with computer software SPSS for Windows (version 11). Chi-square test was applied to compare the allelic frequencies between normal controls and prostate cancer patients. The $P < 0.05$ was considered statistically significant. The gene–gene interactions analysis was conformed using Epical 2004 (version 3.2.2).

Results

The allelic variants in the prostate cancer patients have been summarized in Table 2. The total incidence of the genotype containing $-/-$ allele in *ERα* was higher in prostate cancer patients (18%) than in healthy individuals (10%). There was a threefold increased risk for prostate cancer in individuals carrying this genotype as compared to $+/+$ genotype (OR, 2.70; 95% CI, 1.08–6.70; $P = 0.032$). This was even evident when OR was adjusted for age (OR, 2.74; 95% CI, 1.10–6.84; $P = 0.030$). With regard to the $+/-$ genotype, not much difference was revealed between groups of prostate cancer patients (54%) and control individuals (48%) and it was not statistically significant (OR, 1.68; 95% CI, 0.91–3.12; $P = 0.096$).

The prevalence of *Rr* genotype in *ERβ* was higher in prostate cancer patients (8.0%) as compared to controls (5.0%), but it was not statistically significant (OR, 1.65; 95% CI, 0.52–5.23; $P = 0.394$).

Among patients, the frequency of PR A1/A2 genotype was higher (16%) than controls (10%) and individuals carrying this genotype had a twofold higher risk for developing prostate cancer (OR, 1.71; 95% CI, 0.73–3.98; $P = 0.211$). The value was however not statistically significant. This was evident when OR was adjusted for age (OR, 1.74; 95% CI, 0.74–4.05; $P = 0.200$).

The frequency of *CYP19* CT genotype was higher in patients (37%) as compared to controls (20%) and this incidence was statistically significant (OR, 2.28; 95% CI, 1.20–4.35; $P = 0.012$). Stratification of patients according to age resulted in a slightly improved OR in individuals carrying CT compared to CC genotype (OR, 2.35 95% CI, 1.23–4.49; $P = 0.010$). There appeared to be an increased risk of prostate cancer associated with the Arg264Cys substitution in the *CYP19* gene.

Table 3 Distribution of combined allelic variants of *ERα* and *ERβ* gene in relation to *PR*, *CYP19* and *VDR* genotypes among prostate cancer patients and controls

Genotype		Cases	Controls	OR (95% CI)[a]	*P*-value
ERα	*ERβ*				
+/+	RR	24 (24%)	40 (40%)	1.00	
+/+	Rr	4 (4.0%)	2 (2.0%)	3.33 (0.56–19.59)	0.183
+/− + −/−	RR	68 (68%)	55 (55%)	2.06 (1.11–3.82)	0.022
ERα	PR				
+/+	A1/A1	26 (26%)	36 (36%)	1.00	
+/− + −/−	A1/A2	14 (14%)	4 (4.0%)	4.84 (1.43–16.42)	0.011
ERα	CYP19				
+/+	CC	16 (16%)	32 (32%)	1.00	
+/− + −/−	CC	43 (43%)	41 (41%)	2.09 (1.00–4.38)	0.049
+/− + −/−	CT + TT	29 (29%)	17 (17%)	3.41 (1.46–7.96)	0.005
ERβ	CYP19				
RR	CC	53 (53%)	15 (15%)	1.00	
RR	CT + TT	39 (39%)	26 (26%)	0.42 (0.19–0.90)	0.027
Rr	CC	6 (6.0%)	58 (58%)	0.02 (0.01–0.08)	0.001
ERβ	VDR				
RR	TT	52 (52%)	39 (39%)	1.00	
RR	Tt + tt	40 (40%)	56 (56%)	0.53 (0.30–0.95)	0.035

OR, odds ratio; CI, confidence interval

$P < 0.05$ was considered statistically significant

[a] ORs adjusted for age

The VDR tt genotype was not significantly associated with prostate cancer risk (OR, 0.43; 95% CI, 0.13–1.39; $P = 0.160$).

No association of *ERα/β*, *PR*, *CYP19* and *VDR* genotypes with prostate cancer risk was observed in relation to clinical stage (localized versus metastasis) and tumour grade (low grade <7 vs. high grade ≥7) (data not shown).

Combined genotype analysis

In the present case–control study by PCR-SSCP analysis, except some cases, no mutations either in DNA-binding (exons 2 and 3) or in hormone-binding (exons 4–8) domains were found, the association of the *ERβ*, *PR* and *CYP19* genotypes, which was evaluated with *ERα* genotype, is shown in Table 3. The heterozygous (+/−) and homozygous mutant (−/−) genotypes of *ERα* gene were combined as a single genotype and used for combination of this gene with other genes.

When the *ERα* was combined with *ERβ*, *PR*, *CYP19* and *VDR* genes, a few genotypes showed positive association and values are given in Table 3. Similarly, when *ERβ* was combined with *ERα*, *PR*, *CYP19* and *VDR* only three combined genotypes showed positive results (Table 4).

The frequency of *PR* gene for A1/A1 was 26% and 14% for A1/A2 alleles. A single copy of the variant allele, i.e. A1/A2 resulted in an OR of 4.84 (95% CI, 1.43–16.42; $P = 0.011$) as compared to the wild A1/A1 genotype when combined with mutant allele of *ERα* gene.

A threefold higher risk was observed when *ERα* and *CYP19* mutant genotypes were combined. It resulted in an OR of 3.41 (95% CI, 1.46–7.96; $P = 0.005$). The risk was also statistically significant when *CYP19* CC was combined with *ERα* mutant (OR, 2.09; 95% CI, 1.00–4.38; $P = 0.049$) genotype (Table 3). There was no association of the *VDR* genotypes with risk when combined with *ERα* gene.

The analysis of the combined *ERβ* genotypes in relation to different genes is given in Table 4. There was no risk of prostate cancer when *ERβ* was combined with *PR* and *CYP19* genes.

The role of *PR* gene in relation to *CYP19* genotype showed that there was no association of these two genes when combined together. There was a fourfold (OR, 3.72; 95% CI, 0.97−14.15) higher probability of prostate cancer risk in individuals having *PR A1/A2* and *VDR TT* in combined genotype.

A highly significant association was found in the combination of *CYP19* with *VDR* genotypes (Table 5).

Discussion

As many as 85 AR mutations are there in prostate cancer tissues [25], almost all being single-base substitutions due to somatic mutations, rather than germline mutations. These mutations are unequally distributed along the length of the AR and their types vary. In the present case–control

Table 4 Distribution of combined allelic variants of PR gene in relation to *CYP19* and VDR genotypes among prostate cancer patients and controls

Genotype		Cases	Controls	OR (95% CI)[a]	*P*-value
A1/A1	CC	50 (50%)	67 (67%)	1.00	
A1/A1	CT + TT	34 (34%)	23 (23%)	1.98 (1.04–3.77)	0.037
A1/A2	CC	9 (9.0%)	6 (6.0%)	2.01 (0.67–6.01)	0.212
A1/A2	CT + TT	7 (7.0%)	4 (4.0%)	2.34 (0.65–8.45)	0.193
A1/A1	TT	43 (43%)	40 (40%)	1.00	
A1/A1	Tt + tt	41 (41%)	50 (50%)	0.76 (0.42–1.38)	0.374
A1/A2	TT	12 (12%)	3 (3.0%)	3.72 (0.97–14.15)	0.054
A1/A2	Tt + tt	4 (4.0%)	7 (7.0%)	0.53 (0.14–1.95)	0.341

OR, odds ratio; CI, confidence interval

$P < 0.05$ was considered statistically significant

[a] ORs adjusted for age

Table 5 Distribution of combined allelic variants of *CYP19* gene in relation to *VDR* genotypes among prostate cancer patients and controls

Genotype		Cases	Controls	OR (95% CI)[a]	*P*-value
CC	TT	33 (33%)	64 (64%)	1.00	
CC	Tt + tt	26 (26%)	9 (9.0%)	5.60 (2.35–13.32)	0.00008
CT + TT	TT	23 (23%)	11 (11%)	4.05 (1.76–9.32)	0.001
CT + TT	Tt + tt	18 (18%)	16 (16%)	2.18 (0.98–4.82)	0.054

OR, odds ratio; CI, confidence interval

$P < 0.05$ was considered statistically significant

[a] ORs adjusted for age

study by PCR-SSCP analysis, except some cases, no mutations either in DNA-binding (exons 2 and 3) or in hormone-binding (exons 4–8) domains were found and it might be due to the small sample size. The mutations seen in exceptional cases on sequencing were found to be non-sense mutations.

The gene for the *ERα* has three known polymorphisms: PvuII, XbaI and B-variant polymorphisms, which are reportedly associated with receptor expression and altered receptor function and with some disorders including breast cancer, hypertension and spontaneous abortion. All three RFLPs are located in the A/B domain, the transactivating factor 1. It is an important site for stimulating transcription from certain estrogen-responsive promoters. Hernandez et al. [26] evaluated the association of the *XbaI* and *PvuII* ESR1 single nucleotide polymorphisms (SNPs) with the risk of prostate cancer in three different racial/ethnic populations of black, Hispanic white and non-Hispanic men. Allelic frequency was significantly different across ethnic/racial groups for both SNPs. No association was observed between Hispanic and non-Hispanic white men for this SNP. Also there was no association between the PvuII SNP and prostate cancer risk across all groups.

The analysis of *ERα* and detection of the PvuII restriction endonuclease showed that there was an increased risk associated with the −/− genotype with an OR of 2.70 (95% CI, 1.08–6.70; $P = 0.03$) and the result was statistically significant. The present results are in coherence with the observations of Suzuki et al. [27] who had found a significant association of the −/− genotyope of the PvuII site in the *ERα* gene in a study on 101 cases and 114 healthy individuals among Japanese population (OR, 3.44; 95% CI, 1.97–5.99; $P = 0.0028$). While in a case–control study by Modugno et al. [28], which was performed among Caucasian population of 88 prostate cancer patients and 241 male controls, no significant association was found between −/− and +/− genotypes of *ERα* gene. In a population-based case–control study in Sweden, Weiderpass et al. [29] had shown an association of PP (−/−) with a non-significantly decreased risk for endometrial cancer (OR, 0.70; 95% CI, 0.34–1.44) compared with the +/+ genotype. In another study of 1,069 breast cancer and 1,166 controls which was conducted in Urban Shanghai in China, there was an association of Pp (+/−) and pp (−/−) genotypes with an increased risk of breast cancer with age-adjusted ORs of 1.3 (95% CI, 1.0–1.7) and 1.4 (95% CI, 1.1–1.8), respectively, compared to *PP* (+/+) genotype [30]. Nonetheless, it has suggested a possible role for *ERα* in hormone-dependent tissues such as prostate and breast.

Although the frequency of *ERβ* Rr genotype was higher in prostate cancer patients as compared to controls, the *P*-value was not statistically significant. This observation showed that *ERβ* when analysed alone does not play an important role in the risk of prostate cancer in the present series of cases. Consistent with our results are the observations of Fukatsu et al. [31], who had found a non-significant association between *ERβ* gene and prostate cancer risk on 147 Japanese prostate cancer patients and 266 urological controls (OR, 0.73; 95% CI, 0.46–1.16; $P = 0.182$).

A polymorphism in intron G of the human progesterone caused by an Alu insertion was reported to be associated with ovarian carcinoma in a group of 67 patients of pooled German/Irish population [32]. Later on, a G to T substitution in exon 4, causing a valine to leucine change in the hinge region of the receptor, and a synonymous C to T substitution in exon 5, was linked to the Alu insertion [33]. The association of PROGINS with breast cancer was examined in North America (68 patients and 101 hospital controls) and in the south of England (292 patients and 220 healthy volunteers) [34, 35]. The allele frequency of PROGINS was slightly lower in the North American Caucasian breast cancer patients as compared with the hospital controls, but the difference was not statistically significant [36]. No difference between cases and controls was observed in the English study [34].

In the present study, no significant association with risk was found in the carriers of *PR* genotype and also on it combination with *CYP19* genotypes. An association was found with combined genotype of *PR* with *VDR* gene.

To date, several polymorphisms have been found in the *CYP19* gene. One of these, a C to T variation in exon 7 resulting in an Arg264Cys amino acid exchange, has been shown to be very common in Asia [37, 38] and could thus be an important modifier of breast cancer risk in the population in this area. The Arg264Cys polymorphism is located in or near recognition site of *CYP19* gene and thus it might enhance the estrogen synthesis and exposure to endogenous estrogen. However, it is also possible that *CYP19* Arg264Cys polymorphism is in linkage disequilibrium with other important polymorphic sites such as TTTA repeat polymorphism. In particular, alleles containing a high number of intronic TTTA repeats have been found to be over-represented in breast cancer patients as compared to controls [39, 40]. Monenen et al. [41] identified a novel SNP in the *CYP19A1* gene in prostate cancer. Individuals carrying the *CYP19A1* variant alleles had a significantly increased risk for prostate cancer (OR, 2.87; 95% CI, 1.10–7.49; $P = 0.03$). Lee et al. [39] had reported an association of *CYP19* Arg264Cys polymorphism with breast cancer in Korean women (OR, 1.5; 95% CI, 1.1–2.2) especially those consuming alcohol ($P = 0.04$). They demonstrated that *CYP19* might function as a low-penetrance gene in breast cancer genetic susceptibility.

In the present case–control study of north Indian population, an increased risk associated with *CYP19* CT

genotype was found and this association was statistically significant (OR, 2.28; 95% CI, 1.20–4.35; $P = 0.01$). Consistent with our results are the observations of Suzuki et al. [40] who had found an association of *CT* and *TT* genotypes of the *CYP19* gene (OR, 1.77; 95% CI, 1.02–3.09; $P = 0.037$) with prostate cancer risk among Japanese population. In another study, Modugno et al. [28] found that the *CYP19* CT genotype is associated with an increase in the risk of borderline significance (age-adjusted OR, 2.50; 95% CI, 0.99–6.28). These data suggested that *CYP19* gene could be used as an indicator for prostate carcinoma prevention in men in Asia.

In the present case–control study, there was an overall lack of association between the *VDR* TaqI polymorphism and the prostate cancer risk. In a hospital-based case–control study on a subgroup of whites, including 96 cases and 162 controls, Taylor et al. [22] had reported that men with the homozygous tt genotype were at decreased risk of developing prostate cancer compared with the TT or Tt genotypes (tt versus TT + Tt: OR, 0.32; 95% CI, 0.15–0.75; $P < 0.01$). In a larger case–control study among physicians' Health study on 372 incidents prostate cancer cases and 591 controls, Ma et al. [42] had observed no significant association of the investigated two *VDR* polymorphisms (TaqI and BsmI) and prostate cancer risk. Similarly, in a case control study consisting of 222 prostate cancer, 209 benign prostatic hyperplasia (BPH) and 128 male controls among Japanese populations, the TaqI polymorphism did not show any significant association with either prostate cancer or BPH [43]. Polymorphism in the *VDR* gene has been reported to be associated with prostate cancer in Indian population. The genotype frequency distribution between the prostate cancer and the control group was statistically significant ($P = 0.003$). Their study demonstrated that the FF genotype of the VDR gene plays an important role in determining the risk of prostate cancer and could be postulated as a good candidate genetic marker [44]. Blazer et al. [45] had also reported a lack of association between the TaqI genotype and the prostate cancer risk among 77 prostate cancer cases and 183 controls (tt versus TT + Tt: OR, 1.4; 95% CI, 0.7–2.8). A lack of association with VDR TaqI polymorphism was observed by Hamasaki et al. [46] among 115 cases and 133 age-matched controls. Similar observations were made by Figer et al. [47] among Israeli and Suzuki et al. [48] among Japanese patients. In a larger study of 1,870 prostate cancer cases and 2,843 controls on Greece population, comparison of tt and TT genotypes showed no difference in the frequency of these two genotypes in prostate cancer population (OR, 0.88; 95% CI, 0.70–1.10) [49]. The frequency of *VDR* genotype was not found to be significantly different between prostate cancer and male controls among Chinese population [50]. Huang et al. [51] had investigated the association of the BsmI, ApaI and TaqI polymorphisms of VDR gene with prostate cancer risk in a Taiwanese population. No significant association was found between the ApaI and the TaqI polymorphisms and the risk of prostate cancer. Similar observations had been made by other authors [52–55]. No statistically significant association of the *TaqI* polymorphism with prostate cancer was found in a study of 190 prostate cancer patients and 190 age-matched men with BPH (OR, 1.76; 95% CI, 0.90–3.45) [56]. There was no suggestion of an overall effect of TaqI polymorphism with prostate cancer susceptibility in subjects of European descent (OR, 0.97; 95% CI, 0.87–1.08), Asian descent (OR, 0.88; 95% CI, 0.66–1.17) or African descent (OR, 0.94; 95% CI, 0.41–2.17) [49]. In a case–control study of 368 prostate cancer patients and 243 BPH, no association was observed for TaqI polymorphism and risk of prostate cancer [54].

This is the first case–control study to our knowledge to examine the possible role of *ERs*, *PR*, *CYP19* and *VDR* genes in the development of prostate cancer in Indian men patients.

Taken together, these results clearly indicate that *ERα* and *CYP19* genes and to the lesser extent *ERβ*, *PR* and *VDR* genes in relation with other studied genes may be playing a role in prostate cancer risk. The small number of subjects in genotype subgroups may make the magnitude of our risk estimates uncertain; therefore, studies with a larger sample size are needed to clarify the complex interactions among the *ERα*, *ERβ*, *PR*, *CYP19* and *VDR* genes.

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Lack of Involvement of the *GNAS1* T393C Polymorphism in Prostate Cancer Risk in a Japanese Population

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Abstract. *Background: GNAS1 encodes the α-subunit of the G_s protein ($G_{s\alpha}$), which binds GTP and stimulates adenylyl cyclase. Activating mutations lead to somatotroph, thyroid, adrenal and gonadal adenomas or the McCune-Albright syndrome and recently the T399C polymorphism in GNAS1 has been reported to be associated with malignancies. The purpose of the present case-control study with 349 Japanese prostate cancer patients and 203 urological controls was to determine whether the GNAS1 T393C polymorphism is associated with prostate cancer risk. Materials and Methods: The GNAS1 T393C polymorphism was examined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Odds ratios (OR) were adjusted for age using multiple logistic regression analysis with SPSS Medical Pack. Results: The allele frequencies were compatible with the control population in Hardy-Weinberg equilibrium with 80, 169 and 100 for GNAS1 C/C, C/T and T/T, respectively in the patients with prostate cancer, compared with 42, 94 and 67 in the controls. No association between the GNAS1 polymorphism and prostate cancer risk was apparent. The C/C genotype was more frequent among the prostate cancer patients (22.9%) than the controls (20.7%), although without significance (OR, 1.30; 95% CI, 0.80-2.12; p=0.29). Conclusion: This pilot study does not support involvement of the GNAS1 polymorphism in prostate cancer risk.*

Prostate cancer is one of the most common male malignancies in industrialized nations and its incidence is generally rising (1). Both genetic and environmental influences may be involved in its etiology (2), including ethnic background, family history, smoking, and diet. Some epidemiological studies have supported an association between dietary fat, particularly saturated or animal , and prostate cancer risk (2, 3). Ethnicity, which reflects the shared genetic inheritance within a group, is clearly an important factor in determining the risk of prostate cancer (2, 4). Although it is well-known that the incidence of prostate cancer is still 2- to 3-fold higher in American Caucasians than in Asians, the numbers of clinical cases have been increasing yearly in Japan and in 2020 prostate cancer is expected to be the most common male neoplasm, as was the case for the stomach in 1995 (4, 5). In addition, a familial aggregation is evident, and men with a family history of prostate cancer have a 2-to 3-fold higher incidence than the general population (2). Such an influence of inheritance of genes may reflect relatively high penetrance, but most cases involve polymorphisms with low penetrance.

Genetic polymorphisms affecting a number of metabolic enzymes have been found to modulate prostate cancer risk (2, 6-8). The most studied polymorphisms are in steroid hormone-related genes such as the androgen receptor, 5 α-reductase type II (SRD5A2) and cytochrome P450c17α

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(CYP17) (6, 7). There is evidence for a hormonal etiology of prostate cancer involving the action of androgens, required for differentiation and growth of the prostate. Testosterone is synthesized from cholesterol by a series of enzymatic reactions involving CYP17 and is then converted to dihydrotestosterone (DHT) in the prostate by SRD5A2. DHT binds to the androgen receptor (AR), leading to the transactivation of some genes with AR-responsive elements. An improved understanding of the molecular epidemiology of prostate cancer should help define the relevance of new prognostic indices and aid treatment decisions.

Heterotrimeric guanine nucleotide-binding proteins (G proteins), which couple seven transmembrane receptors to adenylyl cyclase and mediate signal transduction across the cell membranes, are composed of three distinct subunits α, β, and γ which are encoded by separate genes (9-11). G proteins are generally defined by their specific α-subunit and are classified into four major classes (G_S, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$) based on amino acid substitution similarities of the α-subunits (10). The stimultory G protein (Gs) is expressed ubiquitously, being required for the activation of adenylyl cyclase and the generation of cyclic adenosine monophosphate (cAMP) in cells, *e.g.* pituitary target cells, in response to several hormones, such as growth hormone-releasing hormone (GHRH) and corticotroph-releasing hormone (CRH). The *GNAS1* gene, being located on chromosome 20q13.3, is a complex locus encoding multiple overlapping transcripts (9). Of these, the α subunit of the G_s ($G_{s\alpha}$) is the most extensively characterized and clinically relevant. Mutations of *GNAS1* may cause either loss or gain of function by inactivating or activating signal transduction, thus leading to the clinical phenotype of either hormone deficiency or excess (9). Inactivating germ-line mutations cause Albright's hereditary osteodystrophy (AHO) andpseudohypoparathyroidism (PHP), while activating mutations lead to somatotroph, thyroid, adrenal and gonadal adenomas or McCune-Albright syndrome (MAS) (9). MAS is classified by the triad of polyostoic fibrous dysplasia, hyperpigmented (café-au-lait) skin lesions, and gonadotropin-independent sexual precocity (12). In particular, amino acid substitutions replacing either Arg 201 or Gln 227 have been identified in a subset of growth hormone (GH)-secreting adenomas characterized by extremely high adenylyl cyclase activity and cAMP levels (13). $G_{s\alpha}$ appears to be the product of a proto-oncogene that is converted into an oncogene, designated *gsp*. However, few McCune-Albright syndrome patients have been reported to have a malignancy (12, 14-16). There is a common silent polymorphism T393C in exon 5 of *GNAS1*, which has been studied in Caucasians and Japanese in relation to hypertension (17-20). Associations with other diseases, including malignancy, have also been reported (21-30).

In the present study, the prevalence of the T393C polymorphism of *GNAS1* in patients with prostate cancer and benign prostatic hyperplasia (BPH) controls were examined to evaluate its influence on the risk of prostate cancer.

Table I. *Clinicopathological data.*

	Patients	Controls	*p*-Value
N	349	203	
Mean age	68.7±7.1	70.6±7.4	0.34
Histology			
P	59 (17.3)		
M	145 (42.3)		
W	138 (40.4)		
Stage			
A or B	212 (60.7)		
C	85 (24.4)		
D	52 (14.9)		
GS			
<5	9 (3.6)		
6	47 (19.1)		
7	149 (60.6)		
8-10	41 (16.7)		
PSA level (ng/ml)			
<4	7 (4.8)		
4-10	72 (49.0)		
10-20	45 (30.6)		
20-50	20 (13.6)		
50<	3 (2.0)		

P: Poorly differentiated adenocarcinoma; M: moderately differentiated adenocarcinoma; W: well-differentiated adenocarcinoma; GS: Gleason score; PSA: prostatic-specific antigen; Stage: A (T_{1a-b}, N_0, M_0), B (T_{1c-2}, N_0, M_0), C (T_{3-4}, N_0, M_0), D ($T_{1-4}N_1M_{0-1}$ or $T_{1-4}N_{0-1}M_1$).

Materials and Methods

Selection of patients and controls. Data for prostate cancer subjects (n=349) and controls (n=203) were collected from the records of the Departments of Urology at Mie University Hospital, Chiba University Hospital, JA Suzuka Central General Hospital and Matsusaka Saiseikai Hospital between 1991 and 2007. All were Japanese men and the prostate cancer patients were all histologically confirmed and characterized in terms of their clinical staging (Tumor-Node-Metastasis system) and grading (The General Rules for Clinical and Pathological Studies on Prostate cancer, 2001 established by the Japanese Urological Association and the Japanese Society of Pathology). The cancer cases were classified as stage A ($T_{1a-b}N_0M_0$), stage B ($T_{1c-2}N_0M_0$), stage C ($T_{3-4}N_0M_0$) or stage D ($T_{1-4}N_1M_{0-1}$ or $T_{1-4}N_{0-1}M_1$) based on the modified Whitmore-Jewett system. One hundred and thirty eight patients (40.4%) had well-differentiated, 145 (42.3%) moderately differentiated and 59 (17.3%) poorly differentiated adenocarcinomas. Regarding clinical staging, 212 (60.7%) were in stage A or B, 85 (24.4%) were in stage C and 52 (14.9%) were in stage D. The control group was composed of BPH patients who were healthy and confirmed to be free of prostate cancer by no elevation of serum PSA and/or a negative biopsy. Retropubic prostatectomy was performed for 99 of the controls, with pathological examination to exclude the presence of prostate cancer. All gave informed consent to participate in this molecular genetic study of prostate cancer. The Ethical Committee of Mie University approved this study. The clinicopathological profiles of the study participants are shown in Table I.

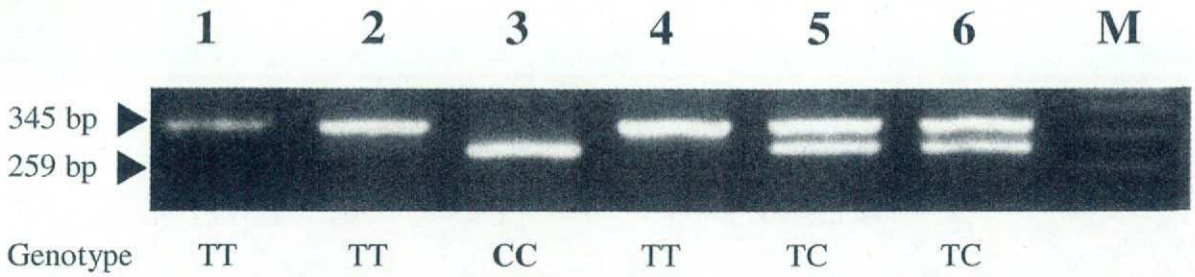

Figure 1. *Representative RFLP patterns of GNAS1 polymorphisms in prostate cancer cases.*

DNA extraction. DNA was isolated from the peripheral blood of all the prostate cancer patients and 104 controls and from the frozen prostate tissue in 99 controls (6).

Genotyping of GNAS1. Genotyping was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. For *GNAS1* T393C, the primers 5'-CTCCTAACTGACA TGGTGCAA-3' and 5'-TAAGGCCACACAA GTCGGGT-3' were used. The PCR products were digested with the restriction enzyme *Fok* I (the T allele produces a 345-bp band; the C allele produces 259- and 86-bp bands). Thus, the unrestricted products (345 bp) represent the TT genotype; the completely restricted products (259 and 86 bp) represent the CC genotype (Figure 1) (29).

Statistical analysis. To examine associations between genotypes and the development of prostate cancer, the odds ratios (ORs) and confidence intervals (CI) were calculated. The ORs were adjusted for age using multiple logistic regression analysis with Dr.SPSS (the Statistical Program for Social Sciences) II for Windows (SPSS, Tokyo).

Results

The mean age of the prostate cancer patients at diagnosis was 68.7±7.1 years and of the controls, 70.6±7.4 years. Although the mean age of the patients was thus lower than the controls, the difference was not significant (p=0.34). The data for the genotypes of the *GNAS1* gene in the prostate cancer patients and controls and their relationships to risk among Japanese males are shown in Table II. The allele frequencies of the *GNAS1* polymorphisms in the patients and controls were compatible with Hardy-Weinberg equilibrium distribution (p>0.05). The frequencies of the *GNAS1* CC, CT and TT genotypes were 22.9%, 48.4% and 28.7% in the prostate cancer patients, compared with 20.7%, 46.3% and 33.0% in the controls. No association between the *GNAS1* CC genotype and prostate cancer risk was apparent (OR 1.30; 95% CI, 0.80-2.12; p=0.29). The OR for prostate cancer risk for men with the CT and CC genotypes was 1.21 (95% CI, 0.81-1.77; p=0.32).

The relationships between the *GNAS1* T393C genotypes and the clinicopathological factors, including histology, staging, Gleason score and serum PSA were investigated (Table III). There was no statistically significant association of the *GNAS1* genotypes with these factors.

Table II. *GNAS1 genotype distribution in patients with prostate cancer and controls.*

Genotype	Patients with prostate cancer	Controls	OR (95% CI)	p-Value
TT	100 (28.7%)	67 (33.0%)	1.00 (referent)	
CT	169 (48.4%)	94 (46.3%)	1.17 (0.78-1.75)	0.45
CC	80 (22.9%)	42 (20.7%)	1.30 (0.80-2.12)	0.29
CT+CC	249 (71.3%)	136 (67.0%)	1.21 (0.83-1.77)	0.32

Results of logistic regression analyses. Age was always included in the models as a covariate.

Discussion

The T393C polymorphism of *GNAS1* is associated with altered $G_{s\alpha}$ mRNA expression in different tissues (21). The T to C substitution at position 393 changes the mRNA folding structures (22), so that genotype-dependent differences in mRNA decay due to an altered secondary structure could be the cause of the variation in $G_{s\alpha}$ mRNA expression (23, 24). *In vitro* studies suggested that increased expression of $G_{s\alpha}$ is associated with enhanced apoptosis and that a second messenger, cAMP, which functions downstream of the G proteins, plays a major role in proapoptotic processes (26, 28, 29). With the reported risk of malignancy, the focus has been on the homozygous CC or TT genotypes. Bladder cancer, colorectal cancer, clear cell renal cell carcinoma, chronic lymphocytic leukemia and squamous cell carcinoma patients with a homozygous CC genotype appear to be at the highest risk of progression, metastasis or tumor-related death, while intrahepatic cholangiocarcinoma and breast carcinoma patients with a homozygous TT genotype showed similar results. In the present preliminary study, the CC genotype was more frequent in the prostate cancer cases, but this was not significant (OR=1.30; 95% CI, 0.80-2.12; p=0.29).