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Restoration of IGFBP-rP1 Increases Radiosensitivity and Chemosensitivity in Hormone-refractory Human Prostate Cancer

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ABSTRACT

We previously reported the tumor-suppressive activity of insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) through induction of apoptosis in human prostate cancer cells. The aim of this study was to investigate the effects of IGFBP-rP1 for radiosensitivity and chemosensitivity in hormone-refractory human prostate PC-3 cancer cells. Five assays were performed using PC-3 cells transfected with IGFBP-rP1 (PC-3rP1) and control cells transfected with an empty vector (PC-3N): PC-3rP1 and PC-3N were compared by clonogenic survival assay, cell cycle analysis and apoptotic assay for radiosensitivity. The number of colonies of PC-3rP1 cells significantly decreased after 4 and 8 Gy of irradiation, compared with those of PC-3N in the clonogenic survival assay. After 16 hr irradiation at 8 Gy, the percentage of apoptotic cells significantly increased in PC-3rP1 compared with PC-3N. Growth of PC-3rP1 was significantly lower than that of PC-3N after docetaxel treatment both *in vitro* and *in vivo*. These results indicate that restoration of IGFBP-rP1 to PC-3 cells increases both their radiosensitivity and chemosensitivity.

Key words: *IGFBP-rP1, Prostate cancer, Docetaxel, Radiosensitivity*

About 230,110 new cases of prostate cancer were diagnosed in 2004 in the United States and about 29,900 men were expected to die of the disease in the same year, based on estimates from the American Cancer Society.

Androgen blockade and radiotherapy or total prostatectomy is the first treatment for prostate cancer. Initially, most patients are responsive to androgen blockade in the early stages of the treatment, but many become refractory to this treatment and develop recurrent disease, invasion and metastasis, which lead to death. Chemotherapy using docetaxel is thought to be the standard treatment for hormone refractory prostate cancer, but the effect is not sufficient. Thus, there is an urgent need for a targeted molecular therapy based on the mechanism of the development of prostate cancer.

Insulin-like growth factor (IGF) has an important role in the occurrence and progression of various malignant tumors, including hepatic, prostate and breast cancers[3,23)]. IGF-binding proteins (IGFBPs) are a family of homologous proteins that modulate the actions of IGF in endocrine, paracrine, and autocrine settings through action as positive or negative regulators of the IGF signaling pathway[6)]. In addition, IGFBPs may also have IGF-independent actions. Six distinct IGFBPs that bind IGF with high affinity have been described[1,9,10,12,14,21)]. They share an overall protein sequence identity of 50% and contain 16 to 18 conserved cysteines in the NH_2- and COOH-terminal regions[22)].

We have recently shown that the protein product of human mac25 cDNA, which is structurally related to the IGFBP family and contains the

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"IGFBP motif" (GCGCCXXC) in its NH_2 terminus, specifically binds to IGF, although with relatively low affinity, and constitutes another member of the IGFBP family, IGFBP-7[11,19]. This finding suggests the possible existence of a family of low-affinity IGFBPs, distinct from the high-affinity members, and together these may constitute an IGFBP superfamily. This group of low-affinity IGFBP genes have been classified as IGFBP-related proteins (IGFBP-rPs)[11,19]. IGFBP-rP1 is preferentially expressed in normal leptomeningial and mammary epithelial cells, and in fibroblast cells. It has also been referred to as IGFBP-7, mac25 and prostacyclin stimulating factor[16,19,26]. IGFBP-rP1 has a tumor suppressor- like function and its expression may indicate a disease with a more favorable status, indicating that IGFBP-rP1 exerts a growth suppressive effect on the tumor.

As a result, IGFBP-rP1 restoration in hormone-refractory human prostate cancer cells may provide a new therapy for gene therapy.

This study was to investigate the possibility that IGFBP-rP1 restoration enhances radiosensitivity and chemosensitivity.

MATERIALS AND METHODS

Cell culture

The human prostate cancer cell line PC-3 was obtained from the American Type Culture Collection (Manassas, VA). PC-3 cells were incubated in OPTI-MEM-II medium containing 5% FBS in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Transfection

The procedure of transfection to PC-3 cells was described previously[17].

Briefly, PC-3 cells were used as parent cells and cultured in OPTI-MEM supplemented with FBS and 5 ng/ml kanamycin at 37°C under 5% CO_2. The IGFBP-rP1 cDNA was obtained from a cDNA library made from normal liver tissue. A 1.1-kb IGFBP-rP1 cDNA fragment containing the full-length coding sequence was ligated and cloned into an IRES2 neo-expression vector (Clontech Laboratories, Inc., Palo Alto, CA). The PC-3 cells were transfected by electroporation with IRES2 neo- expression vector bearing the full length wild-type IGFBP-rP1 cDNA (PC-3-rP1). PC-3 cells transfected with a vector not bearing IGFBP-rP1 cDNA served as control cells (PC-3N). Both PC-3rP1 and PC-3N were selected after 36 hr by G418 treatment (200 μg/ml; Promega, Madison, WI) for 5-7 days until all the non-vector-transfected PC-3 cells were dead. Surviving transfected clone cells were maintained with G418 (200 μg/ml) and the formation of individual colonies was monitored. Visible colonies were cloned by the penicillin-cup method and each colony was transferred to a new well in a 12-well tissue culture plate. These cells were cultured in OPTI-MEM supplemented with FBS and 5 ng/ml kanamycin at 37°C under 5%CO_2. Expression of IGFBP-rP1 was checked by northern blotting and western blotting.

Irradiation and Clonogenic Survival Assay

The effect of radiotherapy and radioisotope treatment on human prostate tumor cell survival was investigated using a clonogenic tumor cell assay. Irradiation was performed at room temperature in a ^{60}Co irradiator (Isotron-21, Shimazu Corp.) at a dose rate of 0.5 Gy/min. Control and PC-3rP1 cells were grown in 9-cm dishes until approximately 80% confluent. After each dish was irradiated at 0, 4 or 8 Gy, the cells were plated at 1,000 cells per well. The cells were returned to an incubator after irradiation and maintained at 37°C for 14 days until treated further or harvested. Colonies were stained with crystal violet and surviving colonies were counted.

Apoptosis Assay

Apoptotic cells were detected using an annexin V-FITC Apoptosis Detection Kit (BD PharMingen, San Diego, CA) by flow cytometry using a Becton Dickinson FACS system. Flow cytometric analysis was performed on cells double-stained with Annexin V-FITC and propidium iodide using a FACS Calibur flow cytometer (Becton Dickinson). Briefly, control and PC-3rP1 cells were irradiated with single doses of 0 and 8 Gy, harvested, washed with ice-cold PBS, and then suspended in annexin V binding buffer. The cells were then stained for 15 min at room temperature in the dark and analyzed in groups of 10,000 cells on a FACS Calibur flow cytometer using CellQuest software.

Cell Cycle Analysis

To examine early effects on cell cycling, control and PC-3rP1 cells were irradiated with single doses of 0 and 8 Gy and the dishes immediately returned to the incubator until harvested for flow cytometry at 24 hr after irradiation. Cell cycle analysis was performed using flow cytometric evaluation of DNA content. After harvesting by trypsinization, cells were collected in 1x PBS and centrifuged. The cells were fixed in 70% ethanol and stored at -20°C until further processing. Before flow cytometry analysis, the cells were centrifuged and washed once in PBS. Each sample was prepared according to Protocol of Apo-Direct™ Flow Cytometry Kit for Apoptosis (Chemicon International) and measured in a FACS Calibur flow cytometer and the cell cycle distribution was analyzed using Modefit cell cycle analysis software (Verity Software House, Topsham, ME).

MTT Assay

The *in vitro* growth-inhibitory effects of docetaxel on PC-3 and PC-3rP1 cells were assessed using an MTT assay. Briefly, 1×10^3 cells were seeded in each well of 96-well plates and allowed to attach overnight. The cells were then treated with 0.1 μM docetaxel for 1 hr. After days 1, 2 and 3 of incubation, 20 μl of conditioned medium (CellTiter 96Aqueous One Solution Cell Proliferation Assay; Promega Corp.) was added to each well and incubation was continued for 4 hr at 37°C under 5% CO_2. Plates were analyzed using an ELISA plate reader (Bio-Rad, Hercules, CA) at 570 nm with a reference wavelength of 630 nm.

Treatment of Tumors with Docetaxel

Male athymic mice (BALB/C Slc-nu, 7 weeks old; SLC Japan, Inc., Tokyo, Japan) were housed at 22°C with a 12-hr light, 12-hr dark cycle. Mice were bilaterally, subcutaneously injected with 1×10^6 PC-3N and PC-3rP1 cells/tumor in 250 μl of PBS in the back, and divided randomly into control and docetaxel groups of five mice each. On day 1, 25 mg/kg docetaxel was injected i.p. into the mice in the docetaxel group. Tumor size was measured with calipers weekly over a period of 5 weeks, and tumor volume was estimated using length × width^2.

Statistical Analysis

The difference in values between the two groups was analysed using the Mann Whitney-U and Wilcoxon's test. p of < 0.05 was considered statically significant.

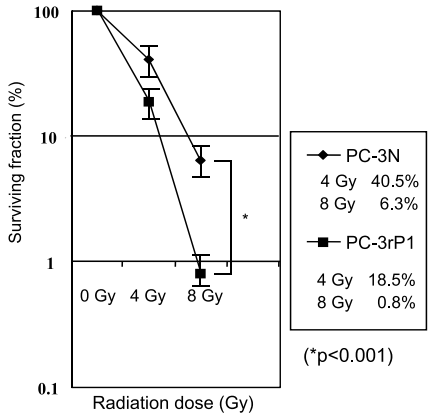

Fig. 1. Irradiation and Clonogenic Survival Assay
Clonogenic cell survival after gamma-ray irradiation. Colonal PC-3 cells transfected with IGFBP-P1 cDNA (PC-3rP1) and those transfected with a control vector (PC-3N) were exposed to 4 or 8 Gy of gamma-ray irradiation and cultured for 14 days. The resulting colonies were stained and counted (> 50 cells). The effects of exposure of PC-3rP1 and PC-3N cells to 4 and 8 Gy of radiation are shown in Fig. 1. The mean survival fractions of PC-3N cells were 40.5% and 6.3% at 4 and 8 Gy, respectively, and those for PC-3rP1 cells were 18.5% and 0.8%, respectively ($p < 0.001$, repeated measures ANOVA). These results indicate that expression of IGFBP-rP1 in PC-3 cells dramatically increased their radiosensitivity.

RESULTS

Increased radiosensitivity of PC-3 cells expressing IGFBP-rP1

PC-3 cells expressing high levels of IGFBP-rP1 (PC-3rP1) and control cells (PC-3N) were prepared

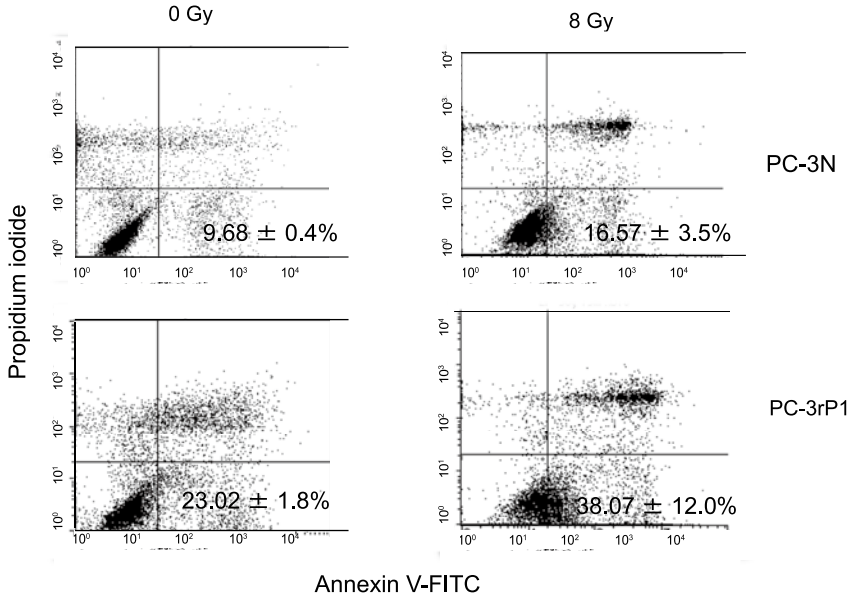

Fig. 2. Apoptosis Assay
Apoptosis was analyzed by Annexin-V/Propidium iodide staining as described in Material and Methods. Apoptotic cells (Annexin-V+/PI-) were detected in the lower right quadrant. Before irradiation, 9.68 ± 0.4% of control cells and 23.02 ± 1.8% of PC-3rP1 cells reacted with the anti-annexin antibody. A time course after irradiation showed that the highest percentages of annexin-positive cells in control cells (16.57 ± 3.5%) and PC-3rP1 cells (38.37 ± 12.0%) were reached at 16 hr after irradiation at 8 Gy.

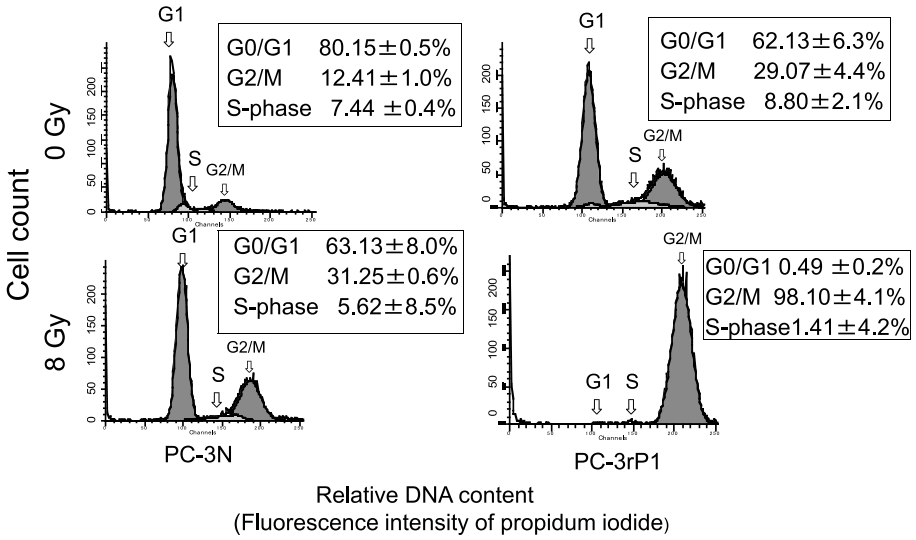

Fig. 3. Cell Cycle Analysis
Effect of IGFBP-rP1 on radiation-induced cell cycle distribution in PC-3 cells. The DNA histogram after 8 Gy or 0 Gy (as a control) of radiation was measured by flow cytometry. The cell cycle arrest in G2/M phase was marked for PC-3rP1 compared with PC-3N in both unirradiated conditions, and after 24 hr exposure to irradiation at 8 Gy ($p < 0.05$ for both).

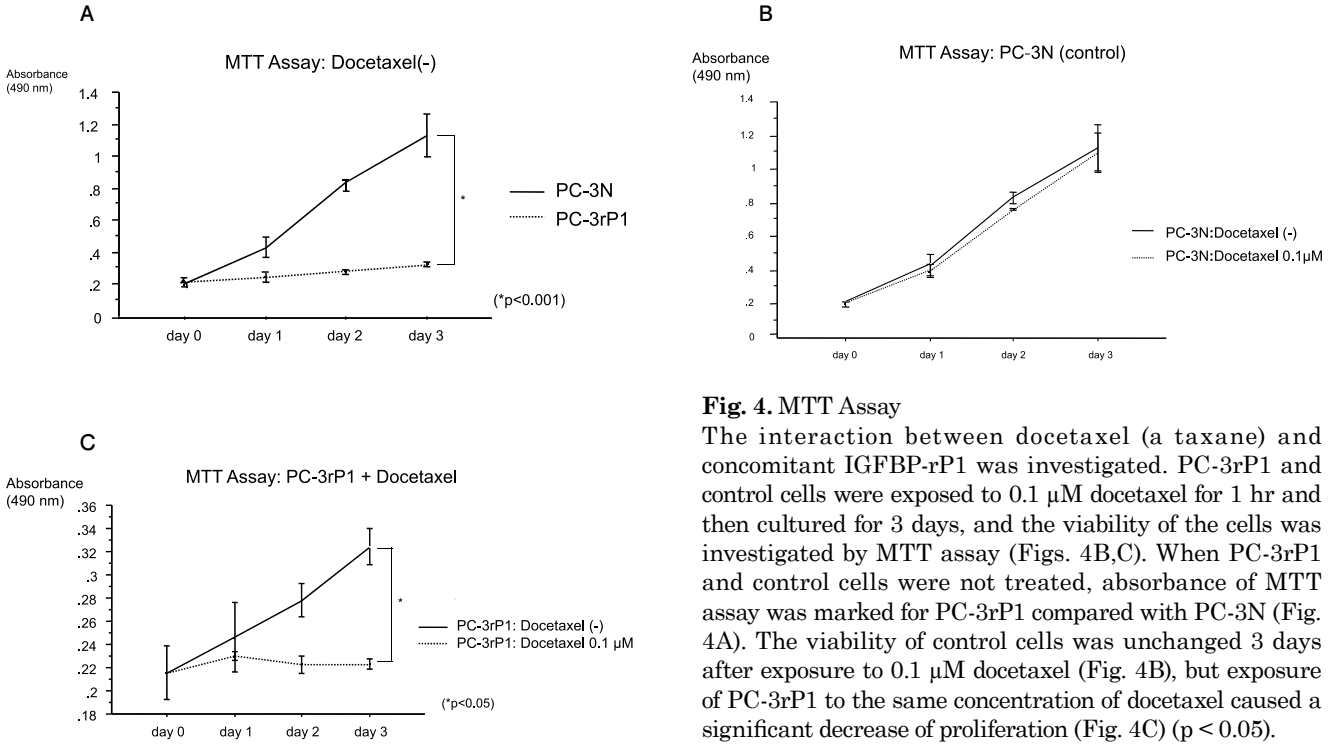

Fig. 4. MTT Assay
The interaction between docetaxel (a taxane) and concomitant IGFBP-rP1 was investigated. PC-3rP1 and control cells were exposed to 0.1 μM docetaxel for 1 hr and then cultured for 3 days, and the viability of the cells was investigated by MTT assay (Figs. 4B,C). When PC-3rP1 and control cells were not treated, absorbance of MTT assay was marked for PC-3rP1 compared with PC-3N (Fig. 4A). The viability of control cells was unchanged 3 days after exposure to 0.1 μM docetaxel (Fig. 4B), but exposure of PC-3rP1 to the same concentration of docetaxel caused a significant decrease of proliferation (Fig. 4C) ($p < 0.05$).

as described in the Materials and Methods. To test the effect of IGFBP-rP1 on population dynamics, clonogenic cell survival was assessed. The effects of exposure of PC-3rP1 and PC-3N cells to 4 and 8 Gy of radiation are shown in Fig. 1. The mean survival fractions of PC-3N cells were 40.5% and 6.3% at 4 and 8 Gy, respectively, and those for PC-3rP1 cells were 18.5% and 0.8%, respectively ($p < 0.001$). These results indicate that expression of IGFBP-rP1 in PC-3 cells dramatically increased their radiosensitivity.

IGFBP-rP1-dependent increase in radiosensitivity is accompanied by increased apoptosis

For investigation of irradiation-induced apoptosis, staining for annexin was performed in PC-3rP1 and control cells at 0, 16, 24 and 32 hr after irradiation at 0, 4 and 8 Gy, and the percentage of cells that reacted with anti-annexin antibody was determined by flow cytometry (Fig. 2). Before irradiation, 9.68 ± 0.4% of control cells and 23.02 ± 1.8% of PC-3rP1 cells reacted with the anti-annexin antibody. A time course after irradiation showed that the

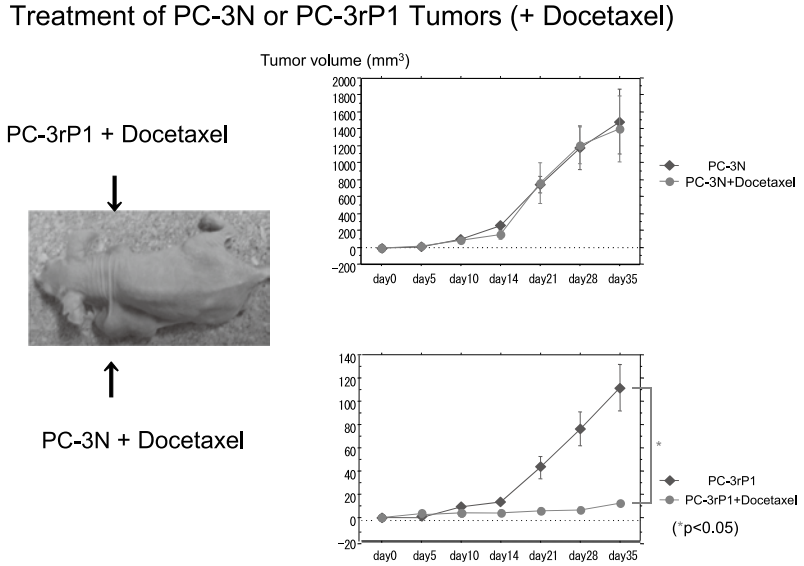

Fig. 5. Treatment of Tumors with Docetaxel
PC-3N and PC-3rP1 cells were subcutaneously injected into nude mice; injection sites were examined in "Materials and Methods." Data points were the mean (± SD) of ten mice. Significance of the difference in tumor size at day 35 was $p < 0.05$.

highest percentages of annexin-positive cells in control cells (16.57 ± 3.5%) and PC-3rP1 cells (38.37 ± 12.0%) were reached at 16 hr after irradiation at 8 Gy ($p < 0.05$). These results indicate that irradiation-induced apoptosis has been more an arithmetic effect between PC-3rP1 and PC-3N.

Cell cycle arrest in G2/M correlates with increased radiosensitivity

The cell cycle was examined at the time of apoptosis induction to investigate DNA damage (Fig. 3). The cell cycle was investigated 12, 24 and 48 hr after irradiation at 0 and 8 Gy. The percentage of cells in G2/M was 98.1 ± 4.1% 24 hr after irradiation at 8 Gy, which was markedly higher than that (31.25 ± 0.6%) in the control cells ($p < 0.05$)[7]. The percentage of cells in the G2/M phase following irradiation damage to DNA was significantly increased by IGFBP-rP1 expression. The result was the same as the apoptosis assay, suggesting the increased radiosensitivity of these cells.

Enhancement of chemosensitivity by IGFBP-rP1 by expression in vitro and in vivo.

The resulting from absorbance of MTT assay was marked for PC-3rP1 compared with PC-3N (Fig. 4A).

PC-3rP1 and control cells were exposed to 0.1 μM docetaxel for 1 hr and then cultured for 3 days, and the viability of the cells was investigated by MTT assay (Fig. 4B, C). The viability of the control cells was unchanged 3 days after exposure to 0.1 μM docetaxel, cell viability of PC-3rP1 significantly decreased compared with PC-3N after exposure to docetaxel at 0.1 μM ($p < 0.05$).

PC-3N and PC-3rP1 cells were subcutaneously injected into nude mice, followed by intraperitoneal administration of docetaxel, and the tumor volume was measured over time (Fig. 5). There was an effect of docetaxel on PC-3N tumors: PC-3rP1 was significantly smaller than PC-3N ($p < 0.05$), and it significantly decreased after treatment with docetaxel.

DISCUSSION

PC-3 is a representative HRPC cell line that is highly tumorogenic and metastatic in nude mice, and is resistant to commonly used inducers of apoptosis[8,13,24]. This is the first report that indicates that restoration of IGFBP-rP1 to PC-3 cells increases both their radiosensitivity and chemosensitivity. At the present, it is not ever report of indicate that possibility of novel therapy for hormone-refractory human prostate cancer (HRPC).

We previously reported the tumor-suppressive activity of IGFBP-rP1 through induction of apoptosis in human prostate cancer cells[17]. Here we have shown that IGFBP-rP1 significantly sensitizes PC-3 cells to irradiation and docetaxel. Clonogenic PC-3 cells expressing IGFBP-rP1 exhibited a significantly reduced rate of survival compared to control mock-transfected cells, and this was further reduced relative to control cells upon exposure to ionizing radiation. These changes were coincident with a significant increase of apoptosis and cell cycle arrest in the G2/M phase. These results indicate that, even though HRPC cells have lost IGFBP-rP1 expression, functional downstream mechanisms are intact and these signals are capable of sensitizing the HRPC cells to radiation damage through enhanced apoptosis.

Recently, Wajapeyee et al reported that IGFBP7 has a central role in BRAFV600E-medicated senescence and apoptosis in melanoma genesis[25]. Thus, oncogene induced senescence and apoptosis are thought to play important roles in suppressing tumorigenesis by preventing proliferation of cells at risk for neoplastic transformation.

Radiotherapy remains a viable treatment for HRPC, but its efficacy is modest and generally it is only used palliatively for symptoms such as bone pain[5]. Recent phase II clinical studies using molecular targets such as hyperactive epidermal growth factor (EGFR) tyrosine kinase in HRPC have also been disappointing[2,4,27]. For these reasons, combined therapy for HRPC has been examined in many clinical trials[18], and our results indicate that a combination of radiotherapy and IGFBP-rP1 expression may be a promising treatment strategy for HRPC.

Anticancer drugs, especially taxanes, act on the polymerization of tubulin and depolymerization of microtubules, which are essential for cell division. Therefore, inhibition of microtubular formation and function by taxane arrests cell division. After completion of cell division, microtubules are depolymerized and return to free tubulin. Taxanes bind to tubulin and promote polymerization to microtubules, as well as binding to tubulin on polymerized microtubules and inhibiting depolymerization. These events lead to excess formation and stabilization of microtubules, and these effects of taxanes may be effective for cases that are non-responsive to microtubule formation-inhibitory vinca alkaloids[20]. Li et al analyzed gene expression after docetaxel administration in established prostate cancer cell lines (LNCap and PC-3) using microarrays. Docetaxel reduced tubulin expression and enhanced microtubule-associated protein expression, and more genes were induced in a hormone-independent cell line, PC-3. The action of docetaxel is not mediated by the androgen receptor, and its direct action on cell division has been demonstrated at the molecular level, as described above[15].

The results of the study suggest IGFBP-rP1 expression inhibited proliferation of a human hormone-refractory prostate cancer cell line (PC-3) and increased the sensitivity of this cell line to irradiation *in vitro*. *In vitro* and *in vivo* results indicated that a combination of IGFBP-rP1 expression with docetaxel may potentiate the growth-inhibitory effect.

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