Glutathione *S*-transferase Pi mediates proliferation of androgen-independent prostate cancer cells

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Prostate cancers generally acquire an androgen-independent growth capacity with progression, resulting in resistance to anti-androgen therapy. Therefore, identification of the genes regulated through this process may be important for understanding the mechanisms of prostate carcinogenesis. We here utilized androgen-dependent/independent transplantable tumors, newly established with the 'transgenic rat adenocarcinoma in prostate' (TRAP) model, to analyze their gene expression using microarrays. Among the overexpressed genes in androgen-independent prostate cancers compared with the androgen-dependent tumors, glutathione *S*-transferase pi (GST-pi) was included. In line with this, human prostate cancer cell lines PC3 and DU145 (androgen independent) had higher expression of GST-pi compared with LNCaP (androgen dependent) as determined by semiquantitative reverse transcription–polymerase chain reaction analysis. To investigate the roles of *GST-pi* expression in androgen-independent human prostate cancers, GST-pi was knocked down by a small interfering RNA (siRNA), resulting in significant decrease of the proliferation rate in the androgen-independent PC3 cell line. *In vivo*, administration of GST-pi siRNA–atelocollagen complex decreased GST-pi protein expression, resulting in enhanced numbers of TdT mediated dUTP-biotin nick-end labering (TUNEL)-positive apoptotic cells. These findings suggest that GST-pi might play important roles in proliferation of androgen-independent human prostate cancer cells.

Introduction

Prostate cancer is the most common cancer and the second most common cause of death in men in the western world. Tumor growth is initially androgen dependent (1). However, with progression, androgen-dependent prostate cancers may become androgen independent and resistant to hormone therapy (2). So far, there are no effective clinical treatments for androgen-independent prostate cancer.

RNA interference is a powerful tool for downregulation of target gene expression to investigate function (3). Recently, many studies have provided support for RNA interference use as a therapy for human diseases, including cancer (3–5). However, systems for clinical delivery are controversial. Atelocollagen complexed with small interfering RNA (siRNA) is reported to have resistance against nucleases and can be efficiently transduced into cells *in vivo* (6–8). With various routes of administration, siRNA–atelocollagen is effective for

gene silencing and efficient inhibition of tumor growth (6). Thus, the knock down of any key gene that can regulate androgen independence might provide a tool for the treatment of patients with advanced prostate cancers.

The transgenic rat adenocarcinoma in prostate (TRAP) features introduction of the *SV40 T antigen* gene under *probasin* control, and androgen-dependent prostate adenocarcinomas develop in 100% of animals by 15 weeks of age, these being transplantable into the subcutaneous back areas of nude mice (9). In the present study, we finally established androgen-independent transplantable tumors by castration of these mice. To identify genes mediating androgen dependence, we compared gene expression between androgen-independent and -dependent transplantable tumors derived from a TRAP rat prostate cancer, employing microarray analysis. Expression of selected genes upregulated in the androgen-independent nude mice tumors was compared with the expression of human orthologs in prostate cancer cell lines LNCaP (androgen sensitive), DU145 and PC3 (androgen independent) to assess whether data from rodent models can be extrapolated to human cases. Furthermore using the bioluminescent PC3M-luc-C6 (PC3ML) originating from PC3 and siRNA–atelocollagen, effects of downregulation of the overexpressed genes were examined.

In this study, glutathione *S*-transferase pi (GST-pi) was investigated as a gene, which is overexpressed in androgen-independent prostate cancers of both human and rodent models.

Materials and methods

Establishment of an androgen-independent transplantable prostate tumor

Establishment of transplanted tumors. A large prostate tumor developing in a lateral lobe of a 55-week-old TRAP rat was minced in Dulbecco's modified Eagle's medium (Invitrogen Corp., Carlsbad, CA), and tissue fragments of ∼8 mm³ were surgically transplanted to the back subcutaneous area of 5-week-old male nude mice (Nippon SLC, Hamamatsu, Japan). Ten weeks after the implantation, tumors were grafted to new mice.

Establishment of an androgen-independent strain. We obtained 10 mice that carried 13th generation transplantable tumors derived from the prostate carcinoma. Five of them were subjected for surgical castration. The tumors in the castrated group did not grow for several weeks, but started to increase in size at 12 weeks after castration. These tumors were repeatedly grafted to new castrated male mice every 10 weeks.

At euthanasia, tumors were excised and cut into slices of 2–3 mm thickness, then fixed in buffered formalin and routinely processed for embedding in paraffin for histological evaluation with hematoxylin and eosin staining. For immunohistochemical analysis, 3 μm thick sections were cut and incubated with the first antibody, and binding was visualized with a Vectastain Elite ABC kit (Vector Laboratory, Burlingame, CA) and light hematoxylin counterstaining. An SV40 large T antigen antibody (PharMingen, San Diego, CA) and an androgen receptor (AR) antibody (Signal Laboratory, Dedham, MA) were used for confirmation of the transgenic origin.

Total RNA extraction and quantitative reverse transcription–polymerase chain reaction

Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan) and treated with DNaseI (Promega, Madison, WI). One microgram samples were converted to complementary DNA with avian myoblastosis virus reverse transcriptase and oligo dT primers (TaKaRa, Otsu, Japan) in 20 μl of reaction mixture and 2 μl aliquots were subjected to quantitative polymerase chain reaction in 20 μl reactions using CYBR Premix Ex Taq™ (TaKaRa) and a Light Cycler apparatus (Roche Diagnostics, Mannheim, Germany). The fluorescence intensity of double-strand-specific SYBR Green I, reflecting the

amount of formed polymerase chain reaction product, was monitored at the end of each elongation step and cyclophilin messenger RNA (mRNA) levels were employed to normalize for the 5′ sample complementary DNA content. Primer sequences for rat Gstp2 were 5′-GCTCTTTAGGGCTTTATGGG-3′ and 5′-CTGTTTACCATTGCCGTTGA-3′ and for human GST-pi were 5′-TGAAT-GACGGCGTGGAG-3′ and 5′-CCCTCACTGTTTCCCGTTGC-3′.

Atelocollagen and cell lines

Atelocollagen was supplied by Koken (Tokyo, Japan). Details were described in a previous paper (6). Human prostate carcinoma cell lines (PC3, LNCaP and DU145) were cultured in RPMI1640 (Invitrogen Crop. and Gibco, Carlsbad, CA) with 10% fetal bovine serum. The bioluminescent human prostate carcinoma cell line PC3M-luc-C6 (Xenogen, Alameda, CA) was cultured in Eagle's minimum essential medium (Invitrogen Corp.) supplemented with 10% heat-inactivated fetal bovine serum (Equitech-Bio, Kerrville, TX), nonessential amino acids (Sigma–Aldrich, St.Loius, MO), L-glutamine (ICN, AlisoViejo, CA), 1 mM sodium pyruvate (Sigma–Aldrich), minimum essential medium vitamin solution (Sigma–Aldrich) and 200 μg/ml zeocin (Invitrogen Corp.). The cells were maintained in vitro at 37°C in a humidified atmosphere of 5% CO_2.

siRNA preparation

Human GST-pi siRNA (Silencer predesigned siRNA, 2292) was purchased from Ambion (Austin, TX). Silencer negative control #2 siRNA (Ambion) with no significant homology to any known human genes was used as a negative control siRNA. The ability of siRNA to silence GST-pi mRNA expression revel was checked on the second day after transfection.

siRNA transfection and cell growth assay in vitro.

PC3ML cells were plated at 5×10^4 cells per 6 cm well. One day after plating, cells were transfected with 100 nM siRNA using DharmaFECT (Darmacon and GE Healthcare, Buckinghamshire, England) according to the manufacturer's protocol. For monitoring inhibition of growth, cells were lysed (n = 3) on days 2, 4 and 6 and then analyzed for luciferase activity (Bright-Glo Luciferase Assay System, Promega). Data were normalized using results for vehicle-treated cells.

In vivo siRNA transfection and imaging of tumors.

Nude mice-transplanted PC3ML were administered with D-luciferin (150 mg/kg) (Promega) by intraperitoneal injection. Twenty minutes thereafter, photons from animal whole bodies were counted using IVIS (Xenogen) according to the manufacturer's instructions. Details were described in the previous paper (7).

Western blotting

Frozen samples were lysed in LIPA buffer and 20 ml extracts were resolved 12% polyacrylamide gels and transferred to Hybond ECL (GE Healthcare). The following antibodies were used for immunodetection: anti-GST-P (MBL, Nagoya, Japan) and anti-beta actin (Sigma, St Louis, MI).

Statistical analysis

Statistical analysis was performed with the chi-square test and Student's t-test using the StatView-J 5.0 program (Berkeley, CA).

Results

Establishment of an androgen-independent transplantable prostate tumor

A model-transplantable prostate adenocarcinoma line derived from a TRAP rat was established with the methods described in Materials and Methods. The tumors proved able to grow in castrated nude mice, expressed SV40 large T antigen showing the TRAP rat origin and were androgen independent with AR localization in the cytoplasm (Figure 1).

Gstp2 expression in nude mice-transplanted prostate cancers

When mRNA expression data for androgen-independent and androgen-dependent transplantable prostate carcinomas newly established, were simply compared, Gstp2 was found to be 17.9 times overexpressed in the androgen-independent nude mice tumors by microarray analysis using a CodeLink rat whole-genome bioarray in which Gstp1 was not included. The result was confirmed by quantitative reverse transcription–polymerase chain reaction using RNAs extracted from five independent tumors of each type. Expression of Gstp2 mRNA in the androgen-independent group was elevated >17-fold (P < 0.0008) compared with the androgen-dependent group.
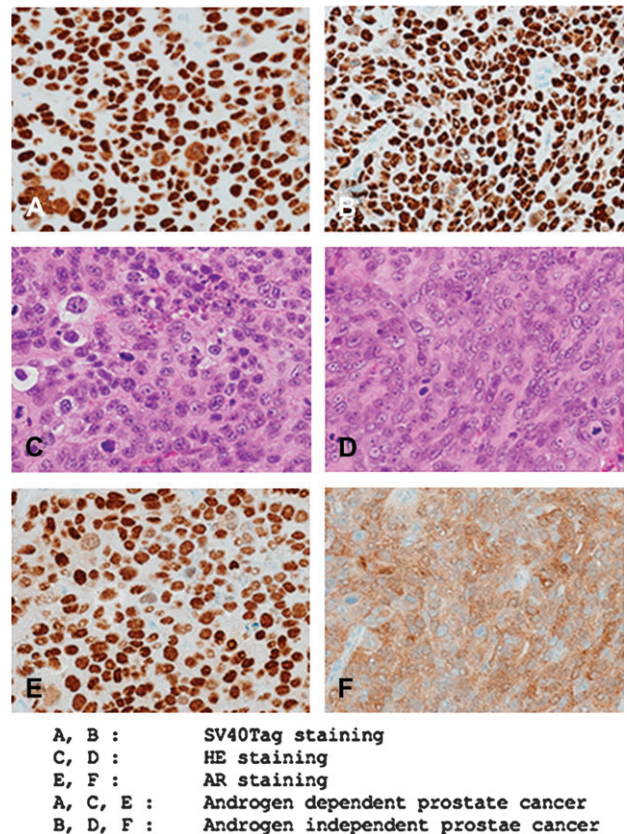

A, B :	SV40Tag staining
C, D :	HE staining
E, F :	AR staining
A, C, E :	Androgen dependent prostate cancer
B, D, F :	Androgen independent prostae cancer

Fig. 1. Representative histological appearance of transplanted prostate cancers in nude mice in the androgen-dependent group (**A**, **C**, **E**) and the androgen-independent group (**B**, **D**, **F**). SV40 large T antigen (SV40Tag) was stained to confirm origin from the TRAP rat prostate tumor (A, B). Hematoxylin and eosin (HE) staining was performed to assess the degree of differentiation (C, D). Castration effects were confirmed by the localization of AR. Non-castrated mice showed nuclear staining of the AR (E), whereas castrated mice showed faint cytoplasmic staining (F), indicating inactive receptor function.

GST-pi expression in human prostate cancer cell lines

We also investigated the expression of human GST-pi, which is a homolog of rat Gstp2 expressed in three human prostate cancer cell lines (LNCaP, PC3 and DU145) and found higher expression in the PC3 and PC3ML cell lines (Figure 2A).

GST-pi expression and proliferation of PC3ML cells

In vitro. To silence GST-pi expression, human GST-pi siRNA was applied into PC3ML cells and as a control, negative control siRNA. As a result, drastic inhibition of proliferation activity was observed only in the GST-pi siRNA case by the imaging anaysis (Figure 2B). This result was confirmed by histological observations. PC3ML showed proliferating many cells becoming full confluence on the dish (Figure 2Ca), but GST-pi siRNA-treated PC3ML cells did not grow at all, showing small number of living cells (Figure 2Cb).

In vivo. To examine the effect of silencing of GST-pi in vivo, PC3ML cells were implanted subcutaneously into the backs of nude mice. Eight mice for each group were administered with GST-pi siRNA–atelocollagen complex or atelocollagen alone. Luciferase signaling levels were slightly decreased, although the result was not statistically significant in the GST-pi siRNA-treated group at day 21 as compared with the control group (Figure 3A and B). Final weights of tumors did not show significant differences (P = 0.72, Figure 3C). We then examined the expression levels of GST-pi in the tumors by western blotting (Figure 3D). Administration of GST-pi siRNA–atelocollagen
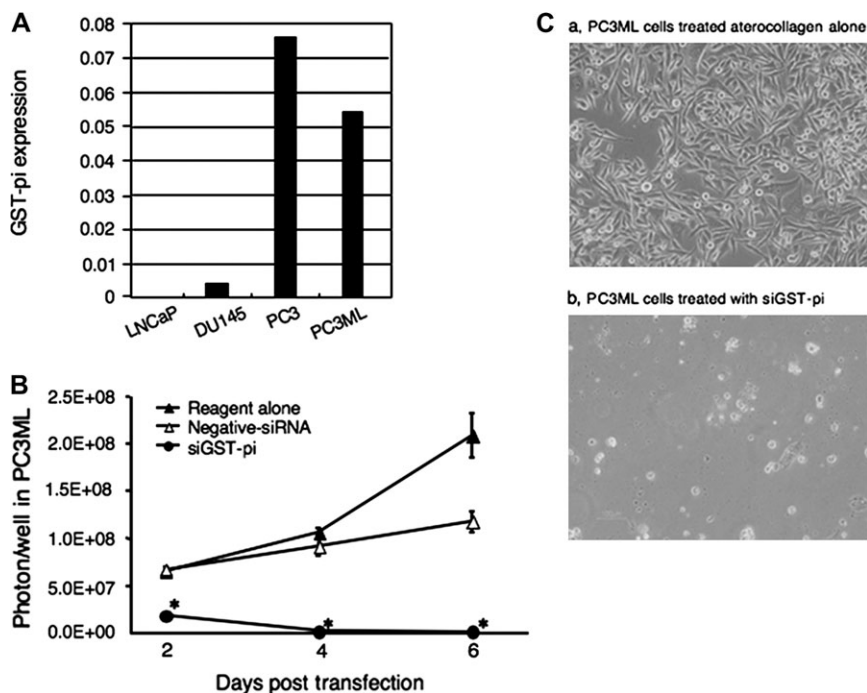
Fig. 2. The androgen-dependent human prostate cell LNCaP does not express GST-pi, in contrast to androgen-independent DU145, and especially PC3 and PC3ML with high expression of GST-pi (**A**). Drastic growth inhibition was detected in the siGST-pi-treated PC3ML with reference to luciferase activity statistically significant (*$P < 0.05$) (**B**) and microscopic appearance with many proliferating cells in PC3ML treated with atelocollagen alone (**Ca**), whereas dead cells in PC3ML treated with siGST-pi–atelocollagen (**Cb**), indicating proliferating activities of GST-pi.

complex increased apoptosis appearance of TUNEL-positive cells compared with atelocollagen alone from 1.271 ± 0.190 to $3.486 \pm 0.450\%$ (mean ± SD) (Figure 3Ea and Eb).

GST-pi staining was apparently observed in the control tumor tissue (Figure 3Ec) in contrast to the siRNA-treated group with negative staining, confirming the effect of GST-pi siRNA–atelocollagen complex effect.

Discussion

In this study, we found that GST-pi might participate in proliferation of the androgen-independent human prostate cancer cell line, PC3. We also showed transplantable tumors (10) in nude mice derived from the TRAP rats to express Gstp2. Mammalian GSTs active in drug metabolism are now classified into the alpha, mu and pi classes (11). Pi-class GSTs have received particular interest in relation to carcinogenesis (12–17). Gstp is well known to be markedly increased in the early stages of rat liver carcinogenesis (18,19). Expression levels of GST-pi have also been found to be elevated in many human tumors (14,16,20,21).

The transplantable tumors in nude mice derived from the TRAP cancer were poorly differentiated adenocarcinomas expressing SV40 large T antigen. They also expressed AR in their nuclei, but once androgen levels were depressed by castration, the localization of AR was changed to the cytoplasm, indicating a lack of function in androgen-independent strains (see Figure 1). The mechanisms were not clear and we tried to find key factors by comparing mRNA expression of androgen-independent and -dependent transplantable tumors. *GST-pi* was found to be the most significantly overexpressed gene in androgen-independent prostate cancers. There are many reports of relations between GST-pi and prostate cancer (14,15,17,21–30) and loss of expression by methylation of their promoter sequences being found as an early event in human prostate carcinogenesis (17,20,31–36). Using the mouse model of knockout Gstp1/Gstp2, Ritchie *et al.* (37) have shown associated enhancement of lung carci-

nogenesis by chemical carcinogens. In skin tumorigenesis, similar effects in the Gstp knockdown mice were reported by Hendersin *et al.* (38).

These results in lung carcinogenesis of mice appear opposite to our finding of growth suppression with decreased Gstp expression. In contrast, GST-pi was reported to promote tumorigenesis of human colon cancer cells (16) similar to the present contribution of GST-pi to proliferation of androgen-independent prostate cancer cells. This contradiction may be due to multiple functions of GST-pi or differences in knockdown methods between mice and rat or human models.

Here, we demonstrated that androgen-independent human prostate cancer cell lines, DU145 and PC3, expressed GST-pi and silencing caused significant growth inhibition of PC3ML cells *in vitro*, but not *in vivo*. The reason may be due to altered cell expression of GST-pi *in vivo* even in the atelocollagen-alone group and low efficiency of the treatment (59.5% GST-pi-positive cells in the atelocollagen-alone group to 49% in the GST-pi siRNA group).

In our results, the suppression due to siGST-pi resulted in statistically significant apoptosis in the treated cells ($P = 0.0005$ by Student's *t*-test) (Figure 3a and b). Inhibition effects on apoptosis by GST-pi in human prostate cell lines were already reported by Lu *et al.*, suggesting an involvement of JNK and p38 activity (39). Arriazu *et al.* (40) reported that expression of Gstp could be a response to oxidative stress during carcinogenesis in the rat prostate. The findings overall suggest that GST-pi might contribute to growth of androgen-independent human and rat prostate carcinomas. The clinical relevance of GST-pi overexpression in prostate carcinogenesis and acquirement of androgen independence should now be clarified *in vivo*.

In summary, we identified Gstp2 in the nude mice model and GST-pi in human prostate cancer cell line PC3 as responsible genes for androgen-independent growth of prostate cancers. To identify more precise mechanisms of androgen-independent growth converting mechanism from androgen dependency, newly established nude mice models of androgen dependent and independent with human prostate cancer cell lines may be useful.
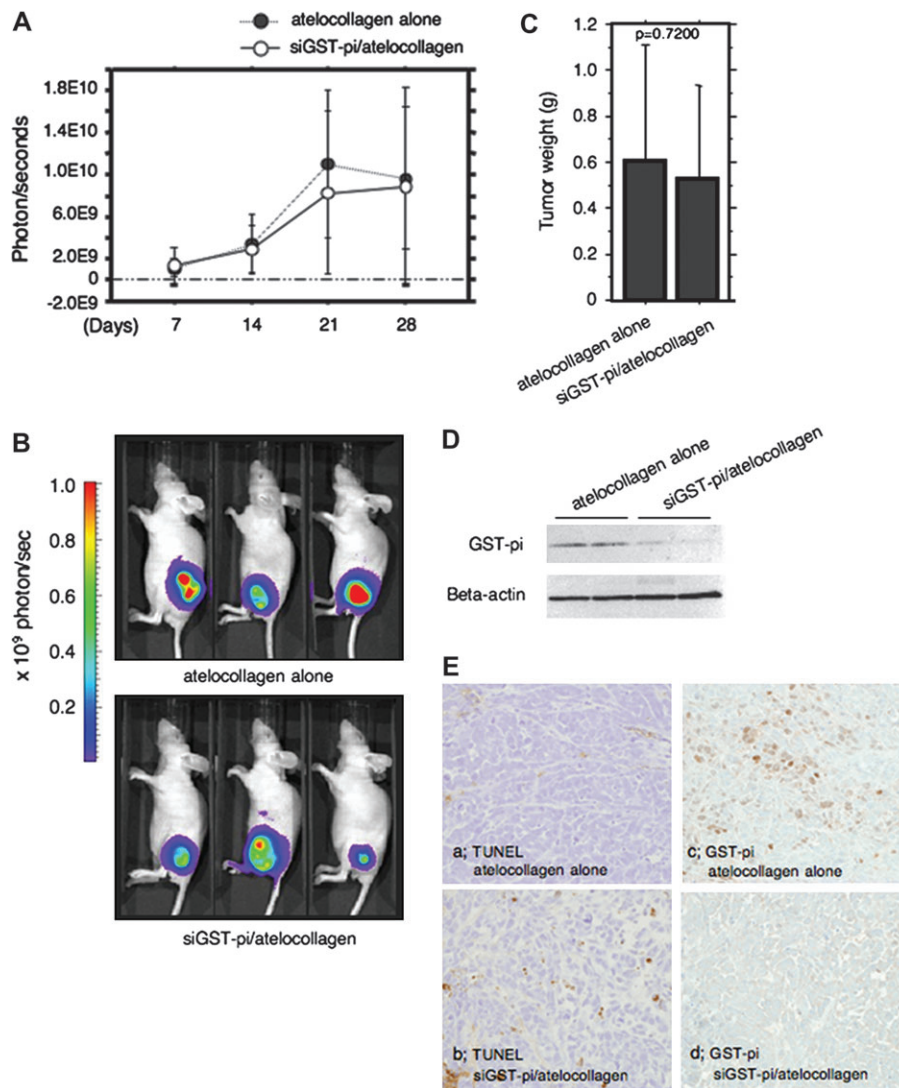
Fig. 3. *In vivo* effects of GST-pi siRNA–atelocollagen complex detected with reference to luciferase signaling (**A** and **B**) and tumor weight (**C**) in PC3ML cells transplanted in nude mice. Inhibitory effects on tumor growth were minimal. However, GST-pi siRNA–atelocollagen complex inhibition of GST-pi expression was confirmed in western blots (**D**). Representative immunohistological staining for TUNEL in the transplanted nude tumors revealed more apoptotic cells in the GST-pi–atelocollagen-treated prostate cancer tissue (**Eb**) than after atelocollagen alone (**Ea**). In the GST-pi immunostaining, apparent, many GST-pi-positive cells were recognized in the control tumor tissue (**Ec**), whereas few GST-pi-positive cells in the siGST-pi–atelocollagen-treated tissue (**Ed**).

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