Biochemical and Biophysical Research Communications 391 (2010) 1075–1079

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Anti-tumor effect of small interfering RNA targeting the androgen receptor in human androgen-independent prostate cancer cells

Koji Azuma [a], Koh-ichi Nakashiro [b,c,*], Toyokazu Sasaki [a], Hiroyuki Goda [b], Jun Onodera [d], Nozomu Tanji [a,c], Masayoshi Yokoyama [a], Hiroyuki Hamakawa [b,c]

[a] Department of Urology, Ehime University Graduate School of Medicine, 454 Shitsukawa, Toon, Ehime 791-0295, Japan
[b] Department of Oral and Maxillofacial Surgery, Ehime University Graduate School of Medicine, 454 Shitsukawa, Toon, Ehime 791-0295, Japan
[c] Department of Cell Growth and Tumor Regulation, Ehime University, Proteo-Medicine Research Center, 454 Shitsukawa, Toon, Ehime 791-0295, Japan
[d] Koken Co., Ltd., 3-14-3 Mejiro, Toshima-ku, Tokyo 171-0031, Japan

ARTICLE INFO

Article history:
Received 3 December 2009
Available online 14 December 2009

Keywords:
Androgen receptor
Atelocollagen
Prostate cancer
RNA interference

ABSTRACT

Early phase prostate cancer is usually androgen-dependent, with the androgen/androgen receptor (AR) signaling pathway playing a central role. At this stage, the cancer responds well to androgen ablation therapy, but prostate cancers eventually acquire androgen independence and more aggressive phenotypes. Several studies, however, have shown that the majority of tumors still express functional AR, which is often amplified and mutated. To determine if the AR is a plausible therapeutic target, we investigated the anti-tumor effect of small interfering RNAs targeting the AR (siAR) in the human prostate cancer cells, LNCaP and 22Rv1, which express mutated AR. In both types of cells, transfection of siAR suppressed mutated AR expression and significantly reduced cell growth. Furthermore, atelocollagen-mediated systemic siAR administration markedly inhibited the growth of 22Rv1 cells subcutaneously xenografted in castrated nude mice. These results suggest that the AR is still a key therapeutic target even in androgen-independent prostate cancer (AIPC). Silencing of AR expression in AIPC opens promising therapeutic perspectives.

Introduction

Prostate cancer is the leading cancer diagnosis and the second most common cause of cancer-related death in men in the United States [1]. Although organ-confined prostate cancer is often curable by surgery or radiation, treatment for locally advanced, recurrent, or metastatic prostate cancer is primarily androgen ablation therapy through surgical or medical castration. Androgen ablation therapy is effective in controlling growth of cancer in approximately 70% of patients [2], but its effects are temporary due to progression of surviving tumor cells to the androgen-independent state [3,4]. At this stage, cancerous cells nevertheless continue to express a transcriptionally active androgen receptor (AR), as evidenced by the expression of androgen-dependent genes such as the prostate-specific antigen (PSA) gene.

Many factors contribute to the development of prostate cancer including somatic mutations of the AR or AR amplification. Some AR mutations result in altered ligand specificity, permitting activation by non-androgenic steroid hormones or even by anti-androgens. AR amplification with concomitant overexpression of the AR can increase the sensitivity of prostate cancer cells to low levels of androgens and eventually result in the development of androgen-independent growth [5]. There are also other mechanisms for developing an androgen-independent state including cross-talk between the AR and other signal transduction pathways, alterations in the expression of steroid co-activators and co-repressors, and androgen-independent mechanisms [6]. Because the AR plays central role to the development of androgen independence, knockdown of AR has been proposed as alternative therapy after failure of androgen ablation therapy.

RNA interference (RNAi) is an endogenous gene-silencing mechanism that involves double-stranded RNA-mediated sequence-specific mRNA degradation [7,8]. RNAi technologies are currently the most widely used techniques in functional genomics. In this study, we investigated the effect of synthetic small interfering RNAs (siRNA) targeting AR (siAR) on the *in vitro* and *in vivo* growth of human androgen-independent prostate cancer (AIPC) cells expressing mutated AR.

Abbreviations: AIPC, androgen-independent prostate cancer; AR, androgen receptor; HMBS, hydroxymethylbilane synthase; IPA, Ingenuity Pathways Analysis; ISGF-3γ, interferon stimulated gene factor 3γ; MX1, interferon-induced myxovirus resistance protein 1; OAS2, 2′,5′-oligoadenylate synthetase 2; RNAi, RNA interference; siAR, siRNA targeting AR; siGFP, siRNA specific for green fluorescent protein; siRNA, small interfering RNA.

* Corresponding author. Address: Department of Oral and Maxillofacial Surgery, Organ Function Regenerative and Reconstructive Medicine, Ehime University Graduate School of Medicine, 454 Shitsukawa, Toon, Ehime 791-0295, Japan. Fax: +81 89 960 5396.
E-mail address: nakako@m.ehime-u.ac.jp (K. Nakashiro).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.12.024

Materials and methods

Cells and cell culture. The human prostate cancer cell lines LNCaP and 22Rv1 were maintained in RPMI1640 (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Biosource, Camarillo, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Immunohistochemistry. Sections (4 μm) of formalin-fixed, paraffin-embedded tissue were deparaffinized and rehydrated. They were treated with 2% H_2O_2 in 50% methanol for 40 min to eliminate endogenous peroxidase, and heated in 10 mM citrate buffer (pH 6.0) for 25 min in a microwave oven to facilitate antigen retrieval. The avidin–biotin–peroxidase complex method with a rabbit Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used. For AR staining, rabbit polyclonal antibody against human AR (N-20; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:300 dilution was applied to sections. The resulting immunocomplexes were detected with 3,3′-diaminobenzidine (Sigma–Aldrich, St. Louis, MO). All sections were counterstained with hematoxylin, dehydrated with ethanol, and cleared with xylene.

Western blot analysis. Cells were grown in monolayers, harvested at subconfluence, and lysed with lysis buffer [0.5 M EDTA and 1% Triton X-100 (Sigma–Aldrich) in PBS containing a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland)]. The samples were centrifuged at 15,000 *g* for 15 min at 4 °C, and supernatants were electrophoresed and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 5% non-fat dried milk (Wako) and 1× TBS-T [25 mM Tris–HCl, 125 mM NaCl, and 0.1% Tween 20 (Sigma–Aldrich)] overnight at 4 °C. They were then probed with polyclonal rabbit anti-human AR antibodies (N-20; Santa Cruz Biotechnology) or monoclonal mouse anti-β-tubulin antibody (BD Biosciences, San Jose, CA) for 1 h, followed by treatment with horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse IgG (Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature. The immune complexes were visualized with the use of the enhanced chemiluminescence (ECL) Plus kit (Amersham Bioscience).

Design and transfection of synthetic siRNAs. We designed and synthesized eight AR-specific synthetic siRNAs. Target sequences were selected using B-Algo and siPRECISE (B-Bridge, Mountain View, CA) for maximum target gene silencing, minimum off-target effects, and the avoidance of single nucleotide polymorphisms (SNPs) and high frequency prostate cancer mutation positions in the AR gene. siRNA specific for green fluorescent protein (siGFP) was used as a negative control. Transfection was performed with Lipofectamine RNAiMAX (Invitrogen) mixed with 10 nM siRNA for Western blotting and 1 nM for the cell proliferation assay.

Cell proliferation analysis. Cells (5×10^4) were seeded into 60-mm dishes in complete medium with synthetic siRNA and Lipofectamine RNAiMAX (Invitrogen) and incubated for 4 days. Cells were recovered by treatment with 0.05% trypsin–0.53 mM EDTA (Wako) and counted with a Z1 Coulter counter (Beckman Counter, Fullerton, CA).

Xenografts model and tumor therapy. Cells (1×10^6) in 0.1 ml culture medium were mixed with 0.1 ml Matrigel (BD Biosciences) and then injected subcutaneously at two sites in the flanks of male athymic nude mice (CLEA Japan, Tokyo, Japan). Two weeks later, tumor cell-bearing nude mice were randomly divided into four treatment groups as follows: no treatment, castration alone, castration and one of two siARs complexed with atelocollagen (AteloGene® Systemic Use; Koken, Tokyo, Japan). Each group consisted of three mice. The final concentration of siRNA was 40 μM in atelocollagen. These complexes were injected into the tail vein every 3 days. Tumor diameters were measured at regular intervals with digital calipers, and tumor volumes (mm^3) were calculated using the formula (length × width × height × 0.523). After 15 days from the first administration of siAR/atelocollagen complexes, 22Rv1 xenografts and several organs (liver, kidney, lung) were dissected and submitted for further analysis. The excised 22Rv1 xenografts were examined for AR expression levels by Western blotting. Total RNA was extracted from several organs, and tested to determine whether the atelocollagen-mediated siRNA systemic delivery induced interferon responses in mice. The animal experiments in this study were performed in compliance with guidelines of the Institute for Laboratory Animal Research, Ehime University Graduate School of Medicine, Japan.

Real-time quantitative reverse transcriptional polymerase chain reaction (RT-PCR). The relative quantitation of mRNA levels using the comparative CT method (ΔΔCT method) was carried out by real-time quantitative RT-PCR, with the use of TaqMan probe and primers (Applied Biosystems, Foster City, CA). Hydroxymethylbilane synthase (HMBS) mRNA was used as an internal control. PCR amplification was performed in a 10-μl final reaction mixture containing 5 μl 2× Quantitect RT-PCR Master Mix, 0.1 μl Quantitect RT mix (Qiagen, Hilden, Germany), 0.5 μl probe and primers, and 1 μl total RNA. Thermal-cycling conditions comprised an initial step at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 5 s. The TaqMan probe and primers for interferon-response genes, 2′,5′-oligoadenylate synthetase 2 (OAS2), interferon-induced myxovirus resistance protein 1 (MX1), interferon stimulated gene factor 3γ (ISGF-3γ), and HMBS were purchased from Applied Biosystems. The 5′-fluorescent reporter dye fluorescence was detected with the LightCycler (Roche Diagnostics).

RNA isolation and microarray. Total cellular RNA was purified from 22Rv1 cells with the use of the RNA isolation kit (Isogen; Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. RNA samples from cells treated with two siAR, along with an appropriate control, were submitted for analysis. After the RNA quality check was performed, 1 μg total RNA was used to generate double-stranded cDNA. The cRNA was transcribed with digoxigenin-labeled nucleotides (Roche Diagnostics), fragmented and hybridized to the Human Genome Survey Array (Applied Biosystems) according to the manufacturer's instructions. After washing each chip, the signal was developed with the use of a chemiluminescent detection kit (Applied Biosystems). Processed chips were scanned by a 1700 chemiluminescent microarray analyzer (Applied Biosystems) and results were analyzed with the use of GeneSpring GX 7.3 (Agilent Technologies, Santa Clara, CA) and Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, www.ingenuity.com) software. Functional analysis of IPA identified biological functions or diseases that were most significant to the data set. Fischer's exact test was used to calculate a *P*-value determining the probability that each biological function or disease assigned to that data set was due to chance alone.

Statistical analysis. One-way ANOVA was used to determine the significance of the differences between groups. $P < 0.05$ was considered statistically significant.

Results

Expression of AR protein in benign and malignant epithelial cells of the prostate

Prostatic tissues were obtained from 21 patients with hormone-naïve prostate cancer, hormone-refractory prostate cancer or benign prostatic hyperplasia at Ehime University Hospital. Expression of the AR protein by immunohistochemical staining was positive in all cases at various levels (Supplementary Fig. S1).

RNAi and growth inhibitory effects of siAR

We used RNAi to clarify the function of AR in the cell proliferation of prostate cancer, especially AIPC. We selected eight target sequences as depicted in Table 1. First, we examined whether the human androgen-dependent prostate cancer cells, LNCaP, can grow under AR suppression. Several synthetic siARs markedly reduced the expression of the AR protein (Fig. 1A) and significantly inhibited the *in vitro* growth of LNCaP cells (Fig. 1B). Next, using four of eight siARs that had high RNAi effects on LNCaP cells (siAR-1, 4, 6, and 8), we examined AR-positive and androgen-independent 22Rv1 cells. Three of these siARs also reduced the expression of the AR protein and suppressed the *in vitro* growth of these cells (Fig. 1C and D). These results suggest that the growth of LNCaP and 22Rv1 cells is supported by the AR.

Effect of siAR on the in vivo growth of androgen-independent prostate cancer cells

We assessed siAR-mediated growth inhibition *in vivo* using a mouse model. We administered siAR/atelocollagen complexes into mouse tail veins every 3 days for a total of five injections. We found that these complexes significantly reduced the size of subcutaneously xenografted 22Rv1 tumors, compared with control groups (Fig. 2A). Furthermore, the expression of the AR in excised tumor tissues was notably suppressed in siAR/atelocollagen complex administration groups compared with control groups (Fig. 2B).

The administration of siRNAs may induce adverse effects by activating interferon-stimulated genes involved in the stress response, and causing the non-specific inhibition of cell growth. To evaluate interferon responses, total RNAs derived from the liver, lung, and kidney were analyzed from each group. The induction of interferon-response gene (OAS2, MX1, and ISGF-3γ) expression was examined by real-time quantitative RT-PCR. While a strong response was observed following the injection of the double-stranded RNA (poly I:C) administered intraperitoneally as a control, the siARs (siAR-4 and 6) did not activate these genes at all (Supplementary Fig. S2).

Molecular mechanisms of the anti-tumor effects by AR knockdown

To understand the molecular mechanisms involved in the growth inhibitory effect of siARs, we conducted an oligonucleotide-based microarray analysis after transfection of siARs (siAR-4 and 6) into 22Rv1 cells. The total number of genes differentially up-regulated by more than fivefold in 22Rv1 cells transfected with both siARs was 224, whereas that differentially down-regulated by more than fivefold was 298. Among these, cancer-related genes were identified by functional analysis of IPA (Table 2). Knockdown of AR induced the expression of the tissue inhibitor of metalloproteinase 4 (TIMP4) and the deleted in colorectal carcinoma (DCC), a putative tumor suppressor gene, and reduced the expression of insulin-like growth factor 1 receptor (IGF1R) and matrix metalloproteinase 2 (MMP2), which have oncogenic functions.

Table 1
Target sequences of synthetic siAR.

siRNA	Target sequence
siAR-1	CCGAGGAGCUUUCCAGAAU
siAR-2	CCAAAGGGCUAGAAGGCGA
siAR-3	CCGAAGAAGGCCAGUUGUA
siAR-4	GGACAUGCGUUUGGAGACU
siAR-5	CGGGAAGUUUAGAGAGCUA
siAR-6	CUACCGAGGAGCUUUCCAGAAUCUGUU
siAR-7	UUACACCAAAGGGCUAGAAGGCGAGAG
siAR-8	CACGGGAAGUUUAGAGAGCUAAGAUUA

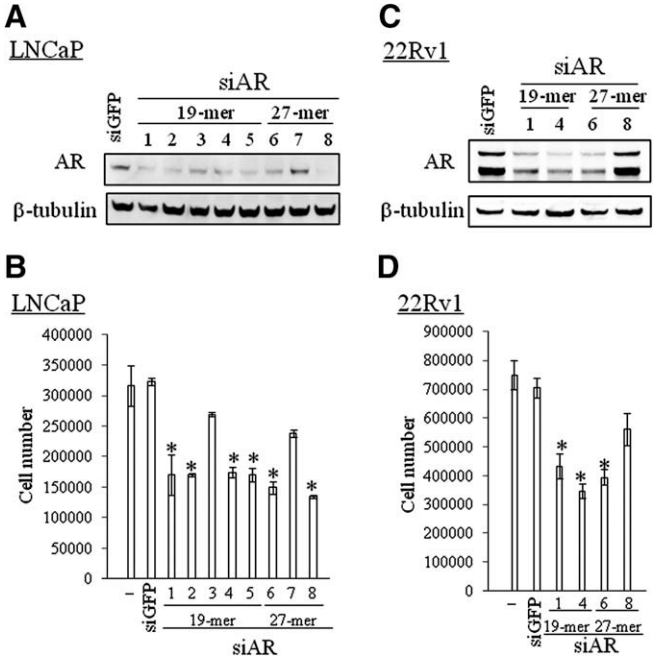

Fig. 1. RNAi effects of siARs in human AR-positive prostate cancer cells. (A) 10 nM siARs were transfected into the androgen-dependent prostate cancer cell line LNCaP with Lipofectamine RNAiMAX. RNAi effect on AR protein expression was evaluated by Western blotting. (B) LNCaP cells (5×10^4) were seeded in complete medium with synthetic siRNAs. Six siRNAs significantly inhibited LNCaP growth. Transfection of 1 nM control siRNA (siGFP) had no inhibitory effect. (C) Three siRNAs (siAR-1, 4, and 6) suppressed the expression of AR proteins in 22Rv1 cells, an AR-positive and androgen-independent prostate cancer cell line. (D) Inhibition of 22Rv1 cell growth. Bars denote SD of samples performed in triplicate. $^*P < 0.01$ compared to control culture.

Discussion

At the time of diagnosis, 10–20% of prostate cancer cases are already in an incurable stage [9]. Among radically treated cases, approximately 30% of them recur [10–12]. Androgen ablation therapy is currently the standard therapy for advanced or recurrent

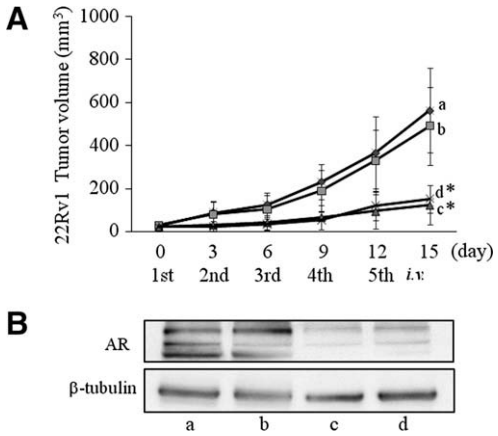

Fig. 2. The effect of siARs with atelocollagen on the *in vivo* growth of 22Rv1. (A) 22Rv1 cells (1×10^6) in equal volumes with Matrigel were injected *s.c.* into the flank of nude mice. Both siAR/atelocollagen complexes were intravenously administered into the tail vein and shown to significantly reduce the growth of 22Rv1 tumors *in vivo*. $^*P < 0.01$ compared to control. (B) The expression levels of AR in the excised tumors were examined by Western blotting. siARs reduced the expression of AR proteins in the tumor tissues. a, no treatment; b, castration alone; c, castration and siAR-4/atelocollagen; d, castration and siAR-6/atelocollagen.

Table 2
Cancer-related genes regulated by AR knockdown in 22Rv1 cells.

Gene symbol	Gene name	Fold change	*P*
Genes up-regulated in AR knockdown cells			
TIMP4	Tissue inhibitor of metalloproteinase 4	63.910	0.00773
AXIN2	Axin 2	13.830	0.0154
STX2	Syntaxin 2	12.800	0.00773
PADI4	Peptidyl arginine deiminase, type IV	10.320	0.0381
MFI2	Antigen p97 identified by monoclonal antibodies 133.2 and 96.5	10.120	0.0184
DCC	Deleted in colorectal carcinoma	8.271	0.0154
PIM1	Pim-1 oncogene	7.445	0.0154
KCNH2	Potassium voltage-gated channel, subfamily H, member 2	6.995	0.0381
MYH11	Myosin, heavy chain 11, smooth muscle	6.849	0.00838
HLA-G	Major histocompatibility complex, class I, G	6.646	0.0306
DNMBP	Dynamin binding protein	5.966	0.0154
MAPK8IP3	Mitogen-activated protein kinase 8 interacting protein 3	5.576	0.00773
BCR	Breakpoint cluster region	5.354	0.00773
IL5	Interleukin 5	5.130	0.00773
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4	5.049	0.0184
Genes down-regulated in AR knockdown cells			
PRSS2	Protease, serine, 2	−52.710	0.00789
INHA	Inhibin, alpha	−50.000	0.0109
IGF1R	Insulin-like growth factor 1 receptor	−33.960	0.00789
AGT	Angiotensinogen	−32.740	0.00789
RPL23A	Ribosomal protein L23a	−30.790	0.00992
MMP2	Matrix metalloproteinase 2	−25.600	0.00128
GPR182	G protein-coupled receptor 182	−15.390	0.0199
ITGB4	Integrin, beta 4	−15.350	0.00128
RECK	Reversion-inducing-cysteine-rich protein with kazal motifs	−14.640	0.00761
IGFBP7	Insulin-like growth factor binding protein 7	−11.930	0.0199
FBLN1	Fibulin 1	−9.714	0.00761
PLAC1	Placenta-specific 1	−8.314	0.0217
HINFP	Histone H4 transcription factor	−7.458	0.0109
TRA@	T cell receptor alpha locus	−7.117	0.0217
THBS1	Thrombospondin 1	−5.367	0.00128

prostate cancer [13,14], but no remarkable advances have been made in the treatment of AIPC.

The AR plays an important role in the proliferation of AIPC. Several mechanisms have been proposed; there is a reportedly large difference (10,000-fold) in the sensitivity in AR between androgen-dependent prostate cancer cells and AIPC cells, the latter responding to even minimal amounts of androgen [15]. Excessive AR expression has been reported in xenografts of hormone-refractory prostate cancer [16], while prostate cells themselves have been shown to directly synthesize dihydrotestosterone [17]. In view of these findings, it seems reasonable to state that AIPC is not androgen-independent, but rather requires androgen for its proliferation and that it may still be dependent on AR. Therefore, a possible treatment strategy is the suppression of AR expression and function. In this report, we show that knockdown of AR expression with synthetic siRNAs significantly inhibited the growth of human AIPC cells expressing mutated AR *in vitro* and *in vivo*.

In the strategy using RNAi method, it is important to avoid adverse reactions (interferon responses and off-target effects) and to establish an *in vivo* delivery system. According to previous reports, interferon responses can be avoided by setting the level of the introduced siRNA at 20 nM or less [18] or by using an siRNA not exceeding 30-bp in length [8]. Here, we used siRNAs at the maximum concentration of 10 nM, and adequate RNAi effects were obtained even when 19- or 27-bp siRNA were used. Furthermore, the interferon responses were successfully avoided in this study; none of the genes known to be involved in the interferon responses (OAS2, MX1, and ISGF-3γ) were induced *in vivo*. The off-target effects were probably minimized by the use of the siPRECISE algorithm, which is a high-performance BLAST-like search that can find sporadic mismatches in a target sequence.

In this study, we used atelocollagen as a carrier for the *in vivo* delivery of siRNA. Atelocollagen is a highly biocompatible biomaterial having been used in the field of healthcare as a component of local hemostatic agents and for tissue regeneration and is unlikely to induce immune reactions *in vivo*. Previous reports showed that an siRNA can efficiently reach the target site *in vivo*, without being degraded by nuclease, if combined at an appropriate concentration of atelocollagen [19,20]. Furthermore, atelocollagen-mediated systemic administration of siRNAs specific for enhancer of zeste homolog 2 (EZH2) and phosphoinositide 3′-hydroxykinase p110-alpha-subunit (p110-α) resulted in efficient inhibition of human prostate cancer cell growth [21].

Our data indicate that AIPC (a clinically important type of prostate cancer) is likely to have an activated AR signal transduction system, and that treatment with AR-targeted siRNA/atelocollagen is an effective strategy for the treatment of AIPC. However, the fact that 22Rv1, AIPC cells, can continue to grow even under AR suppression *in vitro* and *in vivo* indicates some other growth supporting mechanism(s) coexists. According to our microarray analysis, up-regulation of axin 2 (AXIN2), pim-1 oncogene (PIM1), and breakpoint cluster region (BCR) [22–25] may be other coexisting growth-support mechanisms, and involved in the cancer progression. Therefore, treatment targeting these genes may offer an additional therapy of prostate cancer.

Acknowledgment

We thank Dr. Ryoichi Oyasu (Department of Pathology, Northwestern University Feinberg School of Medicine) for his comments on the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.12.024.

References

[1] A. Jemal, R. Siegel, E. Ward, Y. Hao, J. Xu, M.J. Thun, Cancer statistics, 2009, CA Cancer J. Clin. 59 (2009) (2009) 225–249.

[2] J.M. Kozlowski, W.J. Ellis, J.T. Grayhack, Advanced prostatic carcinoma. Early versus late endocrine therapy, Urol. Clin. North Am. 18 (1991) 15–24.

[3] C.L. Martel, P.H. Gumerlock, F.J. Meyers, P.N. Lara, Current strategies in the management of hormone refractory prostate cancer, Cancer Treat. Rev. 29 (2003) 171–187.

[4] A. So, M. Gleave, A. Hurtado-Col, C. Nelson, Mechanisms of the development of androgen independence in prostate cancer, World J. Urol. 23 (2005) 1–9.

[5] M.J. Linja, K.J. Savinainen, O.R. Saramäki, T.L. Tammela, R.L. Vessella, T. Visakorpi, Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer, Cancer Res. 61 (2001) 3550–3555.

[6] J.T. Isaacs, W.B. Isaacs, Androgen receptor outwits prostate cancer drugs, Nat. Med. 10 (2004) 26–27.

[7] A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, C.C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, Nature 391 (1998) 806–811.

[8] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.

[9] B.F. Hankey, E.J. Feuer, L.X. Clegg, R.B. Hayes, J.M. Legler, P.C. Prorok, L.A. Ries, R.M. Merrill, R.S. Kaplan, Cancer surveillance series: interpreting trends in prostate cancer – part I: Evidence of the effects of screening in recent prostate cancer incidence, mortality, and survival rates, J. Natl. Cancer Inst. 91 (1999) 1017–1024.

[10] M. Han, A.W. Partin, C.R. Pound, J.I. Epstein, P.C. Walsh, Long-term biochemical disease-free and cancer-specific survival following anatomic radical retropubic prostatectomy. The 15-year Johns Hopkins experience, Urol. Clin. North Am. 28 (2001) 555–565.

[11] L. Potters, E.A. Klein, M.W. Kattan, C.A. Reddy, J.P. Ciezki, A.M. Reuther, P.A. Kupelian, Monotherapy for stage T1–T2 prostate cancer: radical prostatectomy, external beam radiotherapy, or permanent seed implantation, Radiother. Oncol. 71 (2004) 29–33.

[12] A. Pollack, G.K. Zagars, G. Starkschall, J.A. Antolak, J.J. Lee, E. Huang, A.C. von Eschenbach, D.A. Kuban, I. Rosen, Prostate cancer radiation dose response: results of the M.D. Anderson phase III randomized trial, Int. J. Radiat. Oncol. Biol. Phys. 53 (2002) 1097–1105.

[13] W.W. Scott, M. Menon, P.C. Walsh, Hormonal therapy of prostatic cancer, Cancer 45 (1980) 1929–1936.

[14] Immediate versus deferred treatment for advanced prostatic cancer: initial results of the Medical Research Council Trial. The Medical Research Council Prostate Cancer Working Party Investigators Group, Br. J. Urol. 79 (1997) 235–246.

[15] C.W. Gregory, R.T. Johnson Jr., J.L. Mohler, F.S. French, E.M. Wilson, Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen, Cancer Res. 61 (2001) 2892–2898.

[16] C.D. Chen, D.S. Welsbie, C. Tran, S.H. Baek, R. Chen, R. Vessella, M.G. Rosenfeld, C.L. Sawyers, Molecular determinants of resistance to antiandrogen therapy, Nat. Med. 10 (2004) 33–39.

[17] J.A. Locke, E.S. Guns, A.A. Lubik, H.H. Adomat, S.C. Hendy, C.A. Wood, S.L. Ettinger, M.E. Gleave, C.C. Nelson, Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer, Cancer Res. 68 (2008) 6407–6415.

[18] S. Hara, K. Nakashiro, H. Goda, H. Hamakawa, Role of Akt isoforms in HGF-induced invasive growth of human salivary gland cancer cells, Biochem. Biophys. Res. Commun. 370 (2008) 123–128.

[19] T. Ochiya, Y. Takahama, S. Nagahara, Y. Sumita, A. Hisada, H. Itoh, Y. Nagai, M. Terada, New delivery system for plasmid DNA in vivo using atelocollagen as a carrier material: the Minipellet, Nat. Med. 5 (1999) 707–710.

[20] Y. Minakuchi, F. Takeshita, N. Kosaka, H. Sasaki, Y. Yamamoto, M. Kouno, K. Honma, S. Nagahara, K. Hanai, A. Sano, T. Kato, M. Terada, T. Ochiya, Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing in vitro and in vivo, Nucleic Acids Res. 32 (2004) e109.

[21] F. Takeshita, Y. Minakuchi, S. Nagahara, K. Honma, H. Sasaki, K. Hirai, T. Teratani, N. Namatame, Y. Yamamoto, K. Hanai, T. Kato, A. Sano, T. Ochiya, Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen in vivo, Proc. Natl. Acad. Sci. USA 102 (2005) 12177–12182.

[22] B.E. Wang, X.D. Wang, J.A. Emst, P. Polakis, W.Q. Gao, Regulation of epithelial branching morphogenesis and cancer cell growth of the prostate by Wnt signaling, PLoS One 3 (2008) e2186.

[23] A. Valdman, X. Fang, S.T. Pang, P. Ekman, L. Egevad, Pim-1 expression in prostatic intraepithelial neoplasia and human prostate cancer, Prostate 60 (2004) 367–371.

[24] M. Zemskova, E. Sahakian, S. Bashkirova, M. Lilly, The PIM1 kinase is a critical component of a survival pathway activated by docetaxel and promotes survival of docetaxel-treated prostate cancer cells, J. Biol. Chem. 283 (2008) 20635–20644.

[25] O.O. Olabisi, G.M. Mahon, E.V. Kostenko, Z. Liu, H.L. Ozer, I.P. Whitehead, Bcr interacts with components of the endosomal sorting complex required for transport-I and is required for epidermal growth factor receptor turnover, Cancer Res. 66 (2006) 6250–6257.